REMARKS / ARGUMENTS

Upon entry of the present amendments, claims 35 and 39 are currently under consideration in the application. Claims 1-29 and 31-34 are withdrawn from consideration. Claim 30, and 36-38 are canceled without prejudice. Support for amended claim 35 appears at least in original claim 30 as well as at page 9, lines 25-30 in the specification as originally filed. The foregoing amendments were made without any intention to abandon any subject matter, but with the intention that one or more claims of the same, lesser, or greater scope may be pursued in a later application or in a continuation, continuation-in-part, or divisional application. The present amendment does not add new matter.

The following remarks are responsive to objection/rejections raised by the Examiner in a non-final Office Action, dated May 18, 2007.

Claim Rejections -35 U.S.C. § 112, first paragraph—Written Description

The Examiner rejected claim 39 pursuant to 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement. Specifically, the Examiner alleges that the specification does not provide evidence that the claimed biological materials are (1) known and readily available to the public; (2) reproducible from the written description.

Claim 39 is drawn to a method using a monoclonal antibody selected from the group consisting of: α-IR3; 5C3; and MC192.

Applicants respectfully inform the Examiner that the monoclonal antibodies α-IR3 and MC192 are known in the art and are commercially available. For α-IR3, see for example, Kull, F.C. *et al.* (1983) J. Biol. Chem. 258, 6561; for MC192, see for example, Chandler, C.E. *et al.* (1984) J. Biol. Chem. 259, 6882. Both antibodies are commercially available from, for example, Calbiochem® (www.emdbiosciences.com). For the Examiner's convenience, a copy of these references as well as pages from the Calbiochem® catalogue offering the α-IR3 and MC192 antibodies for sale is enclosed herewith (See Appendix).

The monoclonal antibody 5C3 is also previously described and known in the art. See for example, LeSauteur, L. *et al.* (1996) J. Neurosci. 16, 1308 and U.S. Patent no. 6,610,500 issued August 26, 2003, filed December 6, 1996. Applicants note that the protein sequence for the 5C3 antibody is specified in U.S. Patent no. 6,610,500 at Example III on column 16, line 50 to column 17, line 35. A copy of LeSauteur *et al.* is enclosed herewith for the Examiner's convenience.

In view of the foregoing, Applicants respectfully submit that the monoclonal antibodies α-IR3, 5C3 and MC192 are known and either publicly available or capable of being reproducibly isolated without undue experimentation. Therefore a biological deposit is not required for one of ordinary skill in the art to practice the invention. Applicants respectfully request reconsideration and withdrawal of the rejection.

Claim Rejections -35 U.S.C. § 103

The Examiner rejected claims 35 and 39 pursuant to 35 U.S.C. § 103 as allegedly being unpatentable over Saragovi et al (WO 97/21732) in view of Webb *et al.* (US 6,652,864) and Shih *et al.* (Cancer Immunol. Immunother. 1994; 38:92-98).

Applicants traverse the rejection of claims 35 and 39 under 35 U.S.C. § 103 because this rejection is mooted by the present amendment of claim 35.

Applicants have amended claim 35 to recite a method of treating a patient with a tumor by bypassing the p-glycoprotein pump using immunoconjugates as chemotherapeutic agents that can comprise three genus of monoclonal antibodies wherein the immunoconjugate binds to a specified cell surface antigen (e.g., p75, TrkA; and IGF-1R polypeptide) and is internalized into the cell, bypassing the p-glycoprotein pump, to release the chemotherapeutic agent. Applicants' finding that the compounds of the invention bypass the p-glycoprotein pump after binding to tumor cells, and can treat a patient with a tumor by bypassing the p-glycoprotein pump as

claimed herein, was unexpected. Applicants submit that none of the cited references teach or suggest, alone or in combination, bypassing multidrug resistance or a method of treating a patient with a tumor via bypassing the p-glycoprotein pump.

Saragovi *et al.* teach treating tumors with 5C3, including treating a tumor by coupling a cytotoxic agent to the antibody. Saragovi *et al.* do not teach a breakable linker between the cytotoxic agent and the antibody, or treatment of tumor cells by bypassing the p-glycoprotein pump. Webb *et al.* teach a binding agent that binds selectively to a neurotrophin receptor expressed in nerve cells (including 5C3 and MC192 specifically), a cleavable linker and a non-cytotoxic, therapeutic agent. Shih *et al.* teach an immunoconjugate of an anti-CEA antibody to doxorubicin for treating tumors. None of these references teach or suggest, alone or in combination, compounds to bypass the p-glycoprotein pump. Indeed, Applicants note that Webb *et al.* teach conjugates with a therapeutic, non-cytotoxic agent, which could not be used to determine bypass of p-glycoprotein pump. A cytotoxic agent is required to bypass p-glycoprotein pump. Moreover, Webb *et al.* teach delivery of therapeutic moieties specifically to nerve cells. Nerve cells do not have multidrug resistance or a p-glycoprotein pump. Therefore a person of skill in the art could not have a reasonable expectation based on the teachings of Webb *et al.* that the conjugated compounds could be used to treat tumor cells by bypassing the p-glycoprotein pump. Shih *et al.* do teach conjugates linked to a cytotoxic agent, however Applicants note that Shih *et al.* do not target a conventional receptor. Shih *et al.* use an anti-CEA antibody. CEA is not a transmembrane protein, but rather is linked to membranes via a phosphoinositol lipid bridge (PI linker). In contrast the present claims recite conventional transmembrane receptors for growth factors. Thus Shih *et al.* do not target the same type of receptor that is claimed herein, nor do they teach or suggest bypassing the p-glycoprotein pump. Again, a person of skill in the art would not have a reasonable expectation that the conjugates could be used to bypass the p-glycoprotein pump in treatment of tumor cells.

In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. § 103 rejection of the claim.

CONCLUSION

On the basis of the foregoing amendments, Applicants respectfully submit that the pending claims are in condition for allowance and respectfully request the same. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,

Date: 11/19/07

By _____

FOLEY & LARDNER LLP
Customer Number: 48329
Telephone: (617) 342-4088
Facsimile: (617) 342-4001

Michael Yamauchi
Attorney for Applicant
Registration No. 58,468

APPENDIX

Tech Resources
- Data Sheet
- Data Sheet
- Technical Bulletin
- MSDS - English

Other Languages
- Certificate of Analysis

Store
+2°C to +8°C

Ship
Blue Ice

Note: Store and Ship conditions may differ.
See Key

Anti-Nerve Growth Factor Receptor Mouse mAb (192)

All Categories » Calbiochem » Antibodies » Primary Antibodies » Receptors » Other

Anti-NGF-R

Host: Mouse
Isotype: IgG_1
Immunogen: soluble protein from rat PC-12 cell membranes
Form: Liquid
Formulation: In 50 mM PBS, 0.2% gelatin, pH 7.5.
Preservative: ≤0.1% sodium azide
Positive Control: PC12 cells
Negative Control: FR cells
Comments: Recognizes the ~75 kDa NGF-receptor protein in PC-12 cells.

Ref.: Hempstead, B.L., et al. 1989. *Science* **243,** 373. Levi-Montalcini, R., 1987 *Science* 2 Radeke, M.J., et al. 1987. *Nature* **325,** 593. Johnson, D., et al. 1986. *Cell* **47,** 545. Hosan E.M. 1985. *J. Biol. Chem.* **260,** 655. Chandler, C.E., et al. 1984. *J. Biol. Chem.* **259,** 6882 1979. *J. Biol. Chem.* **254,** 5972.

Need additional information about this product? Email our Technical Service department at technical@calbiochem.com

▸ **Related information for this product is available:** Select
Additional information is available from the "Tech Resources" box in the upper left panel of this page.

EMD Chemicals Inc. USD list price is displayed (pricing with local distributors may vary). Nc is based on item availability worldwide.
Sales Office Contact Details

Size	In Stock	Qty
100 µg	Y	Add to Cart

Clone	Species Reactivity	Applicatio
192	rat not human	IF, IP, NOT IB, Nc See Key

Related Literature:

Protein Kinase and Related Tools Brochure

Material Safety Data Sheets:

GR10: Anti-Nerve Growth Factor Receptor Mouse mAb (192) - English

Bulgarian	French	Italian	Portugues
Danish	German	Korean	Spanish
Dutch	Greek	Norwegian	Swedish
Finnish	Hungarian	Polish	

Related Categories:

All Categories » Calbiochem » Antibodies » Primary Antibodies » Receptors » Other

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Conforms to 93/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number

Customer Number

1. Identification of the substance/preparation and of the company/undertaking

Product name : Nerve Growth Factor Receptor (p75LA^{NGFR}) (Ab-1) Monoclonal Antibody

Catalog # : GR10

Chemical formula : N/A

Supplier : Manufactured by EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
(858)450-5558/(800)854-3417
FAX: (858)453-3552

Synonym : Anti-NGF-R

Emergency telephone number : Call Chemtrec®
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name	CAS No.	EC Number	Symbol	R-Phrases
Anti-NGF-R	N/A	Not available.	-	-

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : No specific hazard.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are nitrogen oxides (NO, NO2. .).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions	:	Splash goggles. Full suit. Boots. Gloves. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.
Small Spill and Leak	:	Absorb with an inert material and put the spilled material in an appropriate waste disposal.
Large Spill and Leak	:	Absorb with an inert material and put the spilled material in an appropriate waste disposal.

7. Handling and storage

Handling	:	Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe gas/fumes/ vapor/spray.
Storage	:	Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store below 4°C (39.2°F).
Packaging materials		
Recommended use	:	Use original container.

8. Exposure controls/personal protection

Engineering measures	:	Provide exhaust ventilation or other engineering controls to keep the airborne concentrations of vapors below their respective threshold limit value. Ensure that eyewash stations and safety showers are proximal to the work-station location.
Hygiene measures	:	Wash hands after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
Nerve Growth Factor Receptor (p75LA^{extra}) (Ab-1) Monoclonal Antibody	Not available.

Personal protective equipment

Skin and body	:	Lab coat.
Eyes	:	Safety glasses
Protective Clothing (Pictograms)	:	

9. Physical and chemical properties

Physical state	:	Liquid.
Color	:	Not available.
Molecular Weight	:	Not available.
Solubility	:	Not available.
Flash point	:	Not available.
Explosive properties	:	Risks of explosion of the product in presence of mechanical impact: Not available. Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability	:	The product is stable.
Conditions to avoid	:	Not available.
Hazardous Decomposition Products	:	These products are nitrogen oxides (NO, NO2...).

11. Toxicological information

RTECS #	:	N/A
Local effects		
Skin irritation	:	Not available.
Acute toxicity	:	LD50: Not available. LC50: Not available.
Chronic toxicity	:	Repeated or prolonged exposure is not known to aggravate medical condition.
Other Toxic Effects on Humans	:	Not available. No specific information is available in our database regarding the other toxic effects of this material for humans. Not available. To the best of our knowledge, the toxicological properties of this product have not been thoroughly investigated.

Carcinogenic effects	:	Not available.
Mutagenic effects	:	Not available.
Reproduction toxicity	:	Not available.
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available.
Toxicity of the Products of Biodegradation	:	The product itself and its products of degradation are not toxic.

13. Disposal considerations

Methods of disposal/ Waste of residues/ Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations.

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class	:	Not controlled under ADR (Europe).

Sea

IMDG Class	:	Not controlled under IMDG.

Air

IATA-DGR Class	:	Not controlled under IATA.
Special Provisions for Transport		Not applicable.

15. Regulatory information

EU Regulations

Risk Phrases	:	This product is not classified according to the EU regulations.
U.S. Federal Regulations	:	TSCA: No products were found. SARA 302/304/311/312 extremely hazardous substances: No products were found. SARA 302/304 emergency planning and notification: No products were found. SARA 302/304/311/312 hazardous chemicals: No products were found. SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found. SARA 313 toxic chemical notification and release reporting: No products were found. Clean Water Act (CWA) 307: No products were found. Clean Water Act (CWA) 311: No products were found. Clean air act (CAA) 112 accidental release prevention: No products were found. Clean air act (CAA) 112 regulated flammable substances: No products were found. Clean air act (CAA) 112 regulated toxic substances: No products were found.
HCS Classification	:	Not controlled under the HCS (United States).
State Regulations	:	
WHMIS (Canada)	:	Not controlled under WHMIS (Canada). No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

Flammability	0
Reactivity	0
Personal Protection	A

National Fire Protection Association (U.S.A.)

Notice to Reader

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Monoclonal Antibodies to Receptors for Insulin and Somatomedin-C*

(Received for publication, November 15, 1982)

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Three monoclonal antibodies, designated αIR-1, αIR-2, and αIR-3, were prepared by fusing FO myeloma cells with spleen cells from a mouse immunized with a partially purified preparation of insulin receptors from human placenta. These antibodies were characterized by their ability to immunoprecipitate solubilized receptors labeled with ^{125}I-insulin or ^{125}I-somatomedin-C in the presence or absence of various concentrations of unlabeled insulin or somatomedin-C. αIR-1 preferentially immunoprecipitates insulin receptors and also less effectively immoprecipitates somatomedin-C receptors, while αIR-2 and αIR-3 preferentially immunoprecipitate somatomedin-C receptors, but may also weakly immunoprecipitate insulin receptors.

These three monoclonal antibodies, as well as A410, a rabbit polyclonal antibody, were used to immunoprecipitate insulin and somatomedin-C receptors from solubilized human lymphoid (IM-9) cells and human placenta membranes that had been ^{125}I-labeled with lactoperoxidase. Analysis of the immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that both receptors are composed of α and β subunits. The β subunit of the insulin receptor (immunoprecipitated by αIR-1 and A410) has a slightly more rapid mobility than the corresponding subunit of the somatomedin-C receptor (immunoprecipitated by αIR-2 and αIR-3). Interestingly, the α subunit of the placenta somatomedin-C receptor has a slightly faster mobility than its counterpart from IM-9 cells.

Immunoprecipitation of receptor that had been reduced and denatured to generate isolated subunits indicates that αIR-2 and αIR-3 interact with the α subunit of the somatomedin-C receptor while A410 interacts with both subunits of the insulin receptor. αIR-1 failed to react with reduced and denatured receptors.

Insulin and somatomedin-C[1] are structurally related peptide hormones with overlapping biological activities (1). Each binds with high affinity to its own receptor,[2] but each can also bind with considerably lower affinity to the other's receptor (2–5). This cross-reactivity is attributable to structural similarities between the receptors as well as between the peptides themselves. Both receptors are composed of two types of subunits, which have approximate molecular weights of about 135,000 and 90,000 (3, 4, 6–12). These are thought to form disulfide-linked heterotetramers containing two copies of each type of subunit (3, 4, 6–8, 10–12). Antibodies from a patient with insulin resistance and acanthosis nigricans have been shown to inhibit the binding of both insulin and somatomedin-C to their respective receptors, suggesting that the receptors are also immunochemically similar (13). Somatomedin-C also binds with relatively high affinity to insulin-like growth factor II receptors. This receptor is structurally different from somatomedin-C and insulin receptors, and has little or no affinity for insulin (3, 4, 14).

The present studies describe three monoclonal antibodies to insulin and somatomedin-C receptors. These are used to investigate the immunochemical cross-reactivity of the two receptors and to identify their subunits in human placenta and IM-9 cells. Some properties of αIR-1 have been described previously (15).

MATERIALS AND METHODS

Receptor Purification—Human placenta membranes were solubilized with 2% Triton X-100, and insulin receptor was purified by sequential chromatography on concanavalin A-Sepharose, insulin-Sepharose, and wheat germ agglutinin-Sepharose (16–18). As previously reported by others (10, 19), we found that somatomedin-C receptors could be quantitatively recovered in the eluate of the concanavalin A column, and that about 30–50% was adsorbed to the insulin-Sepharose column. However, no somatomedin-C binding activity was detected in the urea eluate of the insulin-Sepharose column or at later stages of purification, while insulin-binding activity could be followed throughout the purification procedure (data not shown). In view of results to be presented later, somatomedin-C receptors may have been present but in a denatured form incapable of binding hormone. To assess the degree of purity and the amount of protein present, a small aliquot of the wheat germ agglutinin eluate was reduced and analyzed by SDS[3]-polyacrylamide gel electrophoresis followed by silver staining (20). A 135,000 molecular weight band and faint minor 90,000- and 45,000-molecular weight bands were present. About 5–10 μg of receptor protein was obtained per placenta.

Production of Monoclonal Antibodies—Three SJL mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously, with wheat germ agglutinin-Sepharose eluate containing 3 μg of receptor, emulsified in an equal volume of complete Freund's adjuvant, and boosted three times at 3 week intervals with a similar amount of purified receptor emulsified in incomplete Freund's adjuvant. All mice developed antiserum that immunoprecipitated receptors labeled with ^{125}I-insulin and ^{125}I-somatomedin-C (see Table I for details of assay). The mouse with the highest titer of antibodies to insulin receptors received an i.v. boost of 10 μg of receptor. Three days later, it was sacrificed and its lymph node and spleen cells were fused with FO myeloma cells (Cell Distribution Center, Salk Institute, La Jolla, CA) (21). To achieve an initial cloning stage, hybrids were seeded at a low

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[1] Somatomedin-C has been sequenced and is identical to insulin-like growth factor I (27).

[2] The receptor which is referred to here as the somatomedin-C receptor has also been called the insulin-like growth factor, type I receptor (4). The receptor which is referred to here as the insulin-like growth factor II receptor has also been called the insulin-like growth factor, type II receptor (4).

[3] The abbreviation used is: SDS, sodium dodecyl sulfate.

density using peritoneal exudate cells from SJL mice as feeders (21). Hybridomas grew to numbers exceeding 1000 cells/cm^2/ml of culture medium in 25 out of 1000 wells. Supernatants were screened for antibodies that immunoprecipitated insulin or somatomedin-C receptors as described in the legend to Table I. Six wells were initially positive for antibodies to insulin receptors. These and only these were also positive for antibodies to somatomedin-C receptors. Cell lines from three of these wells eventually died out or stopped producing antibody. Hybridomas from the remaining three wells were serially subcloned by limiting dilution four times. The resulting clones and the antibodies they produce have been designated αIR-1, αIR-2, and αIR-3. Antibodies used in this paper were harvested from ascites fluid of Balb/C × SJL F_1 hybrids (Jackson Laboratories) inoculated with these cell lines. αIR-1 and αIR-3 were further purified on DEAE-cellulose equilibrated with 10 mM potassium phosphate, pH 8.0. αIR-2 was retained on DEAE-cellulose under these conditions, and ascites fluid was used directly without further purification.

All three antibodies are $IgG_1(\kappa)$ (determined by Mono AB-ID EIA Kit, Zymed Laboratories, Burlingame, CA).

Iodination of Cells and Membranes—IM-9 cells were labeled with ^{125}I by using lactoperoxidase (22). The labeled cells were washed with phosphate-buffered saline, solubilized by vortexing with 1% Triton X-100, and the labeled glycoproteins purified by wheat germ agglutinin-Sepharose as described for placenta. Placenta membranes were iodinated with lactoperoxidase as follows: 1 mg of placenta membrane was suspended in 5 ml of phosphate-buffered saline. 100 μg of lactoperoxidase was added followed by 2 mCi of ^{125}I]NaI. A 20-μl aliquot of 10^{-2} M H_2O_2 was added every 4 min for 12 min. The membranes were then washed 3 times by centrifugation at 50,000 × *g* for 30 min with 8 ml of phosphate-buffered saline. The membrane pellet was solubilized with 2% Triton X-100 in 50 mM Tris·HCl, pH 7.7, containing 1 mg/ml of bacitracin and 20 μg/ml of phenylmethylsulphonyl fluoride. After 30 min, the solubilized placenta membranes were centrifuged at 100,000 × *g* for 1 h. The supernatant was diluted with three volumes of Tris·HCl, pH 7.7, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and applied to a 0.5-ml wheat germ agglutinin-Sepharose column equilibrated with this buffer containing 0.2% Triton X-100. The column was washed with 20 ml of the Triton-containing buffer, and the labeled glycoproteins were eluted with 0.5 M *N*-acetyl glucosamine in 50 mM Tris·HCl containing 0.2% Triton X-100, 1 mg/ml of bacitracin, and 20 μg/ml of phenylmethylsulfonyl fluoride.

RESULTS

Table I illustrates the ability of the three monoclonal antibodies to immunoprecipitate receptor-bound ^{125}I-insulin and ^{125}I-somatomedin-C. αIR-1 immunoprecipitates considerably more bound ^{125}I-insulin and ^{125}I-somatomedin-C than does normal mouse serum. If solubilized placenta is omitted from the assay (or if it is heated to 70 °C for 10 min (data not shown)), there is no specific immunoprecipitation of either labeled hormone by αIR-1. This indicates that the antibody is not directly reacting with the hormone (or in the case of somatomedin-C, a binding protein in serum or ascites fluid), but with hormone binding proteins present in placenta membranes. The ability of insulin to inhibit the immunoprecipitation of the labeled hormones indicates that these binding proteins are saturable and have a relatively high affinity for insulin. αIR-2 and αIR-3 both immunoprecipitate more receptor-bound ^{125}I-insulin than normal serum but considerably less than αIR-1. Both antibodies immunoprecipitate similar amounts of bound ^{125}I-somatomedin-C. As with αIR-1, specific immunoprecipitation of both labeled hormones by αIR-2 and αIR-3 is dependent on the presence of solubilized placenta and is inhibited by native insulin, or by heat treating the solubilized placenta (data not shown).

TABLE I

Immunoprecipitation of receptors labeled with ^{125}I-insulin and ^{125}I-somatomedin-C

	cpm Immunoprecipitated (± S.E.)	
	^{125}I-Insulin	^{125}I-Somatomedin-C
Normal mouse serum	112 ± 8	74 ± 11
αIR-1	20,333 ± 362	864 ± 57
αIR-1 + insulin	124 ± 6	117 ± 3
αIR-1 − receptor	115 ± 12	78 ± 6
αIR-2	180 ± 36	1,145 ± 37
αIR-2 + insulin	115 ± 2	402 ± 16
αIR-2 − receptor	121 ± 3	67 ± 8
αIR-3	371 ± 11	816 ± 15
αIR-3 + insulin	128 ± 9	164 ± 15
αIR-3 − receptor	116 ± 6	65 ± 10

Receptor Specificity—Since in these studies, ^{125}I-insulin and ^{125}I-somatomedin-C are immunoprecipitated as labeled hormone-receptor complexes, the potency of unlabeled hormones to compete for receptor binding, and thereby inhibit immunoprecipitation of labeled hormone, reflects their specificity for the receptor. This can be used to identify the receptor to which the labeled hormone is bound when it is immunoprecipitated.

The concentrations of unlabeled insulin and somatomedin-C that inhibit the immunoprecipitation of ^{125}I-insulin by αIR-1 (Fig. 1*A*) are similar to those previously reported to inhibit the binding of ^{125}I-insulin to the insulin receptor (2, 4). This suggests that the ^{125}I-insulin that is immunoprecipitated by αIR-1 (Fig. 1*A*) is bound mainly to the insulin receptor, and that αIR-1, therefore, recognizes insulin receptors. Similarly, the concentrations of unlabeled insulin and somatomedin-C that inhibit the immunoprecipitation of ^{125}I-somatomedin-C by αIR-2 and αIR-3 (Fig. 1, *E* and *F*) are similar to those previously reported to inhibit the binding of ^{125}I-somatomedin-C to the somatomedin-C receptor (2–5). This suggests that αIR-2 and αIR-3 recognize the somatomedin-C receptor.

The competition binding curves in Fig. 1, *B*, *C*, and *D* are more complex. Since ^{125}I-insulin will bind weakly to the somatomedin-C receptor and since αIR-2 and αIR-3 immunoprecipitate the somatomedin-C receptor, it is possible that the relatively small amounts of ^{125}I-insulin immunoprecipitated by these antibodies are bound entirely to somatomedin-C receptors. However, the data (Fig. 1, *B* and *C*) are not consistent with this interpretation. The potency of native insulin to inhibit the immunoprecipitation of ^{125}I-insulin by αIR-2 and αIR-3 is too high, and the potency of unlabeled somatomedin-C is too low (Fig. 1, *B* and *C*) for all the immunoprecipitated ^{125}I-insulin to be bound to the somatomedin-C receptor. Similarly, the potency of unlabeled insulin is too low and the potency of unlabeled somatomedin-C is too high for the ^{125}I-insulin immunoprecipitated by these antibodies to be bound entirely to insulin receptors. The simplest explanation for these data is that ^{125}I-insulin immunoprecipitated by αIR-2 and αIR-3 is bound to a combination of insulin receptors and somatomedin-C receptors. The flat slopes of the competition curves (Fig. 1, *B* and *C*) are consistent with the presence of more than one type of receptor. This reasoning suggests that αIR-2 and αIR-3 do immunoprecipitate insulin receptors, although at the concentration of antibody used, considerably less effectively than αIR-1. Similarly, the ^{125}I-somatomedin-C immunoprecipitated by αIR-1 (Fig. 1*D*) appears to be bound to a mixture of insulin and somatomedin-C receptors, suggesting that αIR-1 weakly recognizes

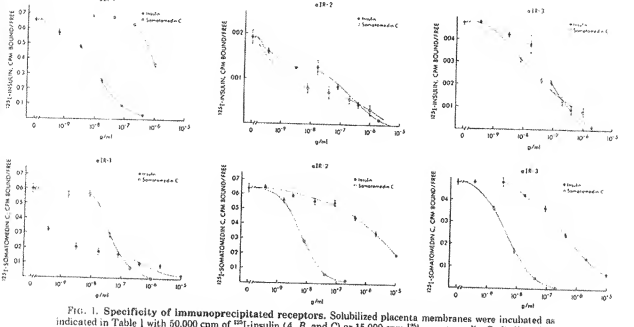

FIG. 1. **Specificity of immunoprecipitated receptors.** Solubilized placenta membranes were incubated as indicated in Table 1 with 50,000 cpm of ^{125}I-insulin (*A*, *B*, and *C*) or 15,000 cpm ^{125}I-somatomedin-C (*D*, *E*, and *F*) in the absence or presence of various concentrations of unlabeled insulin (●) or somatomedin-C (○). The labeled receptors were then immunoprecipitated as described in the legend to Table 1 by: *A* and *D*, αIR-1 (19 μg of IgG/ml); *B*, and *E*, αIR-2 (1:420 dilution of ascites); *C* and *F*, αIR-3 (11 μg of IgG/ml). Nonspecific counts (counts immunoprecipitated by normal mouse serum without added monoclonal antibody) have been subtracted in the calculations.

somatomedin-C receptors.

To further evaluate which receptors are immunoprecipitated by each antibody, placenta membranes were incubated with ^{125}I-somatomedin-C in the absence of unlabeled peptides (Fig. 2, *lanes 1–4*), with 100 ng/ml of somatomedin-C (Fig. 2, *lanes 5–8*), with 100 ng/ml of insulin (Fig. 2, *lanes 9–12*), or with both 100 ng/ml of somatomedin-C and insulin (Fig. 2, *lanes 13–16*). ^{125}I-somatomedin-C was then covalently cross-linked to the receptor to which it was bound with disuccinimidyl suberate. The membranes were solubilized with Triton X-100, immunoprecipitated with normal mouse serum, αIR-1, αIR-2 or αIR-3, and analyzed by SDS-polyacrylamide gel electrophoresis.

αIR-2 and αIR-3 immunoprecipitated a 132,000-*M*, band that was heavily labeled in the absence of somatomedin-C (Fig. 2, *lanes 3* and *4*). Labeling of this band was readily inhibited by 100 ng/ml of somatomedin-C (Fig. 2, *lanes 7* and *8*), but not inhibited by 100 ng/ml of insulin (Fig. 2, *lanes 11* and *12*). Because of its relative affinity for insulin and somatomedin-C and its electrophoretic mobility, this band appears to be the α subunit of the somatomedin-C receptor.

In the absence of unlabeled peptides, the band immunoprecipitated by αIR-1 (Fig. 2, *lane 2*) is less heavily labeled than those immunoprecipitated by αIR-2 or αIR-3. In addition, it is broader and has a portion with a slightly slower electrophoretic mobility. Furthermore, its labeling is only partially inhibited by unlabeled somatomedin-C (Fig. 2, *lane 6*) and is also partially inhibited by unlabeled insulin (Fig. 2, *lane 10*), suggesting that this band is composed of α subunits of both insulin and somatomedin-C receptors.

When similar studies are carried out using ^{125}I-insulin as the labeled peptide instead of ^{125}I-somatomedin-C, αIR-1 specifically immunoprecipitates a labeled band with a molecular weight of 135,000 (Fig. 2, *lane 17*). Labeling of this band is readily inhibited by 100 ng/ml of insulin (data not shown). When ^{125}I-insulin is used as the labeled peptide, αIR-2 and

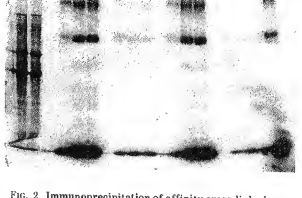

FIG. 2. **Immunoprecipitation of affinity cross-linked receptor.** Placenta membranes (0.4 mg of protein) were incubated for 90 min at 15 °C in 1.0 ml of 20 mM $NaPO_4$, pH 7.4, containing 0.05% albumin with 10^6 cpm of ^{125}I-somatomedin-C (*lanes 1–16*) or ^{125}I-insulin (*lane 17*) and no unlabeled hormone (*lanes 1–4* and *17*), 100 ng/ml of somatomedin-C (*lanes 5–8*), 100 ng/ml of insulin (*lanes 9–12*), or both 100 ng/ml of somatomedin-C and 100 ng/ml of insulin (*lanes 13–16*). Then 0.1 mg of disuccinimidyl suberate was added. After 30 min, the disuccinimidyl suberate was quenched with 20 μl of 1 M NH_4Cl. 4 ml of 50 mM Tris·HCl, pH 7.7, containing 0.2% albumin was added and the membranes pelleted. The membrane pellet was dissolved in 50 mM Tris·HCl containing 2% Triton X-100, with bacitracin (1 mg/ml) and phenylmethylsulfonyl fluoride and centrifuged for 30 min at 200,000 × *g*. The supernatant was diluted 1:4 with Tris·HCl containing bacitracin and immunoprecipitated as described in the legend to Fig. 1 with normal mouse serum (*lanes 1, 5, 9,* and *13*), αIR-1 (*lanes 2, 6, 10, 14,* and *17*), αIR-2 (*lanes 3, 7, 11,* and *15*), or αIR-3 (*lanes 4, 8, 12,* and *16*). The immunoprecipitates were washed twice with 4 ml of Tris·HCl containing 0.2% Triton X-100 and once with 4 ml of H_2O. They were then lyophilized and subjected to SDS-polyacrylamide gel electrophoresis on a 6.5% gel. The standards in the *left lanes* are myosin heavy chain, phosphorylase, and albumin.

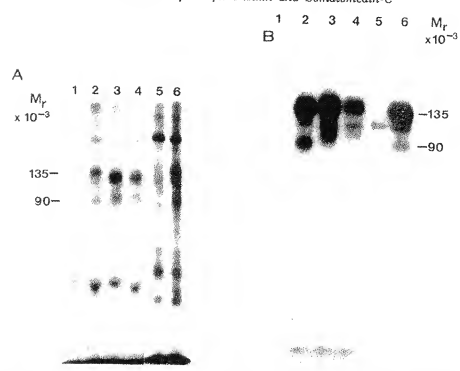

FIG. 3. **Immunoprecipitation of labeled receptor from iodinated human placenta membranes and IM-9 cells.** *A*, ^{125}I-labeled placenta membrane glycoproteins (1.2×10^6 cpm) were incubated in 50 mM Tris·HCl, pH 7.7, containing 0.2% Triton X-100, 0.1% bovine albumin, 1 mg/ml of bacitracin, and 20 μg/ml of phenylmethylsulfonyl fluoride with: *lane 1*, normal mouse serum diluted 1:200; *lane 2*, normal mouse serum diluted 1:300 plus αIR-1 (19 μg of IgG/ml); *lane 3*, normal mouse serum diluted 1:300 plus αIR-2 (ascites fluid 1:420); *lane 4*, normal mouse serum diluted 1:300 plus αIR-3 (11 μg of IgG/ml); *lane 5*, 100 μg/ml of preimmune rabbit IgG; *lane 6*, 85 μg/ml of A410. After 8 h at 4 °C, 20 μl of anti-mouse serum (Cappel) diluted 1:3 was added to the tubes containing mouse immunoglobulin, and 20 μl of fixed staphylococci bearing protein A (Pansorbin) was added to tubes containing rabbit immunoglobulin, and the incubation was continued overnight at 4 °C. The immunoprecipitates were then washed three times with 4 ml of Tris·HCl, containing 0.2% Triton X-100, and once with distilled water. The immunoprecipitates were lyophilized and electrophoresed on a 6.5% SDS-polyacrylamide gel. Shown is an autoradiogram of the dried gel. *B*, ^{125}I-labeled IM-9 cell membrane glycoproteins (3.2×10^5 cpm) immunoprecipitated as described above. *Lane 1*, normal mouse serum; *lane 2*, αIR-1; *lane 3*, αIR-2; *lane 4*, αIR-3; *lane 5*, preimmune rabbit IgG; *lane 6*, A410.

αIR-3 fail to produce detectable specific immunoprecipitation of affinity labeled bands (data not shown). This is consistent with the relatively weak ability of αIR-2 or αIR-3 to immunoprecipitate receptor labeled with ^{125}I-insulin as is indicated by Table I and Fig. 1.

Immunoprecipitation of Lactoperoxidase-labeled Receptors—To further demonstrate that these antibodies interact directly with receptors for insulin and somatomedin-C, and to establish their specificity, we examined their ability to immunoprecipitate ^{125}I-labeled membrane glycoproteins from human placenta and IM-9 cells. As previously described (15), αIR-1 specifically immunoprecipitated two polypeptides with apparent molecular weights of 135,000 and 90,000 from both human placenta and IM-9 cells (Fig. 3*A*, *lane 2* and Fig. 3*B*, *lane 2*). Polypeptides with similar molecular weights were immunoprecipitated by A410 (Fig. 3*A*, *lane 6* and Fig. 3*B*, *lane 6*), a rabbit antiserum to rat liver insulin receptor (23). These bands correspond to the α and β subunits of the insulin receptor described previously by several laboratories (6–9, 22).

αIR-2 and αIR-3 also specifically immunoprecipitate two polypeptides with apparent molecular weights of approximately 135,000 and 90,000 (Fig. 3*A*, *lanes 3* and *4*, and Fig. 3*B*, *lanes 3* and *4*). Because of the specificity of αIR-2 and αIR-3, these presumably are subunits of the somatomedin-C receptor. In both placenta and IM-9 cells, the broad band corresponding to the β subunit has a slightly slower mobility (apparent M_r 92,000–98,000) than the corresponding subunit of the insulin receptor. In some gels, this band appears as a doublet, the faint lower component having a mobility similar to the corresponding subunit of the insulin receptor. Interestingly, in human placenta, the α subunit of the somatomedin-C receptor (immunoprecipitated by αIR-2 or αIR-3) has a slightly faster mobility (apparent M_r 132,000) than the corresponding subunit of the somatomedin-C receptor from IM-9 cells (apparent M_r 136,000) or of the insulin receptor (immunoprecipitated by αIR-1 or A410) from either tissue (apparent M_r 135,000).

In order to determine with which subunit these antibodies interact, immunoprecipitation studies were performed with iodinated placenta membrane glycoproteins that had been treated with dithiothreitol and SDS to dissociate receptor subunits (Fig. 4). After this treatment, neither subunit is immunoprecipitated by αIR-1, perhaps indicating that this antibody recognizes an epitope that is destroyed by reduction and denaturation. αIR-2 and αIR-3 specifically immunoprecipitate the α subunit of the somatomedin-C receptor. A410 immunoprecipitates both the α and β subunits of the insulin receptor. Since A410 is polyclonal, this does not necessarily

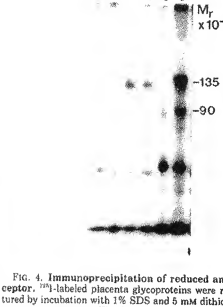

FIG. 4. **Immunoprecipitation of reduced and denatured receptor.** ^{125}I-labeled placenta glycoproteins were reduced and denatured by incubation with 1% SDS and 5 mM dithiothreitol for 5 min at room temperature. The dithiothreitol was then quenched with 12 mM *N*-ethylmaleimide and the denatured proteins diluted 20-fold with 1% albumin in 50 mM Tris·HCl, pH 7.7, containing 0.2% Triton X-100, 1 mg/ml of bacitracin, and 20 μg/ml of phenylmethylsulfonyl fluoride. The reduced and denatured labeled receptor was then immunoprecipitated as described in Fig. 3. *Lane 1*, normal mouse serum; *lane 2*, αIR-1; *lane 3*, αIR-2; *lane 4*, αIR-3; *lane 5*, preimmune rabbit serum; *lane 6*, A410.

imply immunochemically similar sites on both subunits. Similar results were obtained with labeled membrane glycoproteins from IM-9 cells (data not shown).

DISCUSSION

The present studies describe three separate monoclonal antibodies which react predominantly with insulin receptors (αIR-1) or somatomedin-C receptors (αIR-2 and αIR-3). We have interpreted the data in Fig. 1, *B C*, and *D* as indicating that immunoprecipitated labeled ligand is bound to a combination of insulin and somatomedin-C receptors, and therefore, that each antibody can react with both receptors. The ability of αIR-1 to immunoprecipitate both insulin and somatomedin-C receptors is also suggested by affinity cross-linking studies (Fig. 2). However, other explanations for the data are also possible. For example, the antibodies may recognize a third type of receptor that is distinct from both insulin and somatomedin-C receptors and that binds both of these ligands with intermediate affinity. The insulin like growth factor II receptor is a possible candidate, but it probably can be ruled out since that receptor has little or no affinity for insulin (3, 4), while the receptors responsible for labeled ligand binding in Fig. 1, *B*, *C*, and *D* do. Furthermore, polyacrylamide gel electrophoresis of the immunoprecipitates of lactoperoxidase labeled cells and membranes reveals no labeled bands in the 220–260-kDa range (Fig. 3) which could correspond to the insulin-like growth factor II receptor (3, 4, 14).

αIR-2 and αIR-3 have many similar properties. Both are $IgG_1(\kappa)$, both have selectivity for somatomedin-C receptors, and both recognize the reduced and denatured 135,000-molecular weight subunit. However, they are clearly different antibodies. αIR-2 has more stringent specificity for somatomedin-C receptors. (At the antibody concentration used, αIR-2 immunoprecipitates more ^{125}I-somatomedin-C and less ^{125}I-insulin than does αIR-3 (Fig. 1 and Table I)). Furthermore, αIR-2, in contrast to αIR-3, is retained on DEAE-cellulose equilibrated with 10 mM potassium phosphate, pH 8.0 (data not shown).

The structure of insulin receptors has been extensively studied by a variety of techniques (7). It is clearly composed of α and β subunits with molecular weights of approximately 135,000 and 90,000, respectively. Other less well characterized subunits have also been identified by some laboratories (6, 24–26). Some of these may be precursors or degradation products of the receptor (24–26). In Fig. 3, the only detectable bands specifically immunoprecipitated by αIR-1 and A410, which react predominantly with insulin receptors, have molecular weights of approximately 135,000 and 90,000.

Information about the structure of the somatomedin-C receptor is more limited and has been obtained almost exclusively from affinity labeling studies (3, 4, 10, 11). In these studies, a 135,000-molecular weight α subunit has been clearly identified which is disulfide-linked to other subunits. Evidence for a β subunit has been directly inferred from similarities between partially reduced and unreduced forms of the somatomedin-C and insulin receptors (4, 11), although in some affinity labeling studies, a faintly labeled 90,000-molecular weight subunit of the somatomedin-C receptor has been observed (4). The somatomedin-C receptor immunoprecipitated with αIR-2 and αIR-3 (Fig. 3) clearly contains both subunits. The β subunit moves slower on SDS-polyacrylamide gels than the corresponding subunit of the insulin receptor. This is fortunate because it provides a distinct method of distinguishing the two receptors aside from their immunochemical and ligand-binding specificities. In some gels, the β subunit of the somatomedin-C receptor appears as a doublet, the faint lower component having a mobility similar to that of the corresponding subunit of the insulin receptor. The origin of this band is not clear. It may be due to proteolysis or to a small amount of insulin receptor co-immunoprecipitated by these antibodies, or it may be due to a microheterogeneity of somatomedin-C receptor. Interestingly, the α subunit of the somatomedin-C receptor from placenta has a slightly more rapid mobility than its counterpart from IM-9 cells. Here too, this difference may merely result from proteolysis of the receptor during preparation of the membranes, or it may indicate tissue specific differences in the receptors.

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A Monoclonal Antibody Modulates the Interaction of Nerve Growth Factor with PC12 Cells*

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A nerve growth factor (NGF) receptor interactive monoclonal antibody (192-IgG) which enhances β-NGF binding to PC12 cells has been produced. The hybridoma clone was obtained by fusing Sp2/0-Ag14 myeloma cells with splenocytes from Balb/C mice which had been immunized with *n*-octyl glucoside solubilized proteins from isolated PC12 cell plasma membranes. The antibody is an IgG, which does not bind β-NGF. It binds to the same number of sites on PC12 cells at low temperature as does β-NGF. The 192-IgG increases the apparent affinity of β-NGF binding to fast receptors on PC12 cells at low temperature by a factor of 2.5- to 4-fold and enhances the photoactivatable cross-linking of β-NGF to the same receptor while decreasing the cross-linking of β-NGF to the slow NGF receptor. At 37 °C 192-IgG partially inhibits the regeneration of neurites from primed PC12 cells. The 192-IgG also reduces the rate of appearance of binding to slow NGF receptors and increases the proportion of β-NGF bound to fast receptors at 37 °C. These results implicate the slow receptor as the mediator of the biological response. This antibody provides a tool for examining steps in the mechanism of action of β-NGF after binding to the receptor.

NGF[1] is a polypeptide which is required for the development and maintenance of sympathetic and some sensory neurons (1, 2) and is one of the factors involved in the regeneration of sympathetic and sensory axons after injury (3). A specific retrograde flow of NGF occurs from the peripheral target to the neuronal cell body (4). The flow is initiated by the binding of NGF to NGF-Rs in the nerve terminal followed by the internalization of the NGF-receptor complex in membrane-limited vesicles (5). The retrograde flow displays two components, one of high affinity and low capacity and the other of lower affinity and high capacity (6). The NGF-Rs on chick sensory and sympathetic neurons display similar characteristics. One class of NGF-R, present in relatively small numbers, has a high affinity for NGF (K_D of approximately 10^{-11} M) while the other major class has a lower affinity with a K_D of approximately 10^{-9} M (7, 8). Although the process of retrograde flow appears to use both NGF-Rs, NGF-induced neurite outgrowth from sympathetic and sensory neurons, which has a maximal response at concentrations below 10^{-11} M, may be mediated solely by the high affinity NGF-R (9).

The PC12 clonal cell line from a rat pheochromocytoma, which responds to NGF by expressing many of the properties of sympathetic neurons including neurite outgrowth (10), also has two classes of NGF-Rs (11, 12). As with NGF-Rs on sensory and sympathetic neurons their main distinguishing feature is the rate at which NGF dissociates from the receptor, being rapid from the larger class of low affinity receptors and relatively slow from the smaller number of high affinity receptors. The PC12 NGF-Rs have been termed fast and slow receptors, respectively, for this reason (12). Recent evidence (13) suggests that the two classes of NGF-Rs (on sympathetic neurons) may be structurally related. It is known that the low affinity class of NGF binding observed with sensory neurons is not a result of negative cooperativity (7). The conversion from a low affinity (fast) to a high affinity (slow) NGF-R has been suggested for PC12 receptors on the basis of an increased binding to the slow NGF-Rs at the expense of fast NGF-Rs which occurs when ^{125}I-NGF loaded cells are further incubated in the absence of ^{125}I-NGF (11). On the other hand, Schechter and Bothwell (12) have proposed, from experiments on the differential sensitivity of the PC12 cell NGF-Rs to trypsin, that both classes pre-exist on the cell surface. Whether interaction of NGF with its receptors is sufficient by itself to initiate neurite outgrowth is not yet known.

Surface-bound NGF is internalized, especially in PC12 cells, and is degraded in lysosomes (14–16). Inhibition of lysosomal degradation has no effect on the NGF-induced neurite outgrowth (17), nor does the presence within the cell of NGF antibodies prevent neurite outgrowth (18). Although internalized NGF has been reported to appear in a number of other cellular compartments (16, 17, 19) there is no evidence, as yet, that such migration gives rise to intracellular signals for neurite outgrowth. Indeed free NGF inside the PC12 cell is unable to initiate neurite outgrowth (18).

In attempts to probe further the mechanism of action of NGF, particularly with respect to its interaction with the NGF-Rs, a monoclonal antibody which interacts with the NGF-Rs on PC12 cells has been developed. The characteris-

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[1] The abbreviations used are: NGF, nerve growth factor; NGF-R, nerve growth factor receptor; NCS, newborn calf serum; HAT, hypoxanthine, aminopterin, thymidine selective medium; PBS, Dulbecco's phosphate-buffered saline; CMF-PBS, Ca/Mg-free Dulbecco's phosphate-buffered saline; PBS-BSA, Dulbecco's phosphate-buffered saline containing 1 mg/ml of bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSAB, hydroxysuccinimidyl-4-azidobenzoate.

tics of the antibody and its effects on NGF binding and on neurite outgrowth in PC12 cells are described here.

MATERIALS AND METHODS

Cell Culture—PC12 cells (clonal rat pheochromocytoma obtained from D. Schubert, Salk Institute) were grown in Dulbecco's modified Eagle's medium (nos. 430-2100, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA or GIBCO) and 5% horse serum (GIBCO) in a humidified atmosphere of 12% CO_2 and 88% air. Cells were subcultured once per week at a ratio of 1 to 5, and culture medium was changed once per week.

Sp2/0-Ag 14 myeloma cells (obtained from J. Schreurs, Department of Pharmacology, Stanford University) were grown in RPMI 1640 (GIBCO) supplemented with 10% NCS (GIBCO) in a humidified atmosphere of 6% CO_2 and 94% air. Sp2/0 cells were subcultured every 2–3 days by dilution into fresh medium (1/5 to 1/10 dilution).

Hybridoma cells (obtained by fusion of Sp2/0 cells with immune splenocytes, see below) were initially grown in RPMI 1640 plus HAT (20) supplemented with 10% NCS, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. Expansions of initial wells and cloning of hybridoma cells were done with Balb/C thymocyte feeder cells present (10^7 cells/ml). As clones grew, thymocyte feeder cells were omitted. Hybridoma clones were also grown in serum-free medium (HB 101, Irina Biologicals, Berkeley, CA) supplemented with 4 mM glutamine and 1 mM sodium pyruvate. Cells were frozen for storage in growth medium or NCS plus 10% dimethyl sulfoxide.

PC12 Plasma Membrane Preparation—Plasma membranes were prepared from PC12 cells by the method described by Wilson (21). PC12 cells (6×10^8) were washed three times by centrifugation in PBS, pH 7.4, to remove medium and serum components. The cell suspension was divided into three parts for parallel subsequent processing at 4 °C. Each part was resuspended in 10 ml of homogenization buffer (10 mM Tris·Cl, 5 mM $MgCl_2$, 125 μM phenylmethylsulfonyl fluoride (protease inhibitor dissolved in isopropanol; final isopropanol concentration was 0.1%)), pH 7.4, and incubated on ice for 30 min. The suspension was brought to 250 mM sucrose by adding the cell suspension via a 22-gauge needle to an equal volume of 0.5 M sucrose made in homogenization buffer. Cells were immediately homogenized with a motor-driven Teflon/glass homogenizer (Thomas B19250). The homogenate was centrifuged at 1700 × *g* for 10 min. The pellet was rehomogenized in 250 mM sucrose (in homogenization buffer) and again centrifuged at 1700 × *g*. The two low speed supernatants were combined and centrifuged at 33,000 × *g* for 30 min. The medium speed pellets from all 3 parts were combined and resuspended in approximately 15 ml of 250 mM sucrose (in homogenization buffer). Portions of this suspension (2.5 ml) were carefully layered onto each of 6 discontinuous sucrose gradients made in homogenization buffer. The gradients consisted of 6.5 ml of 32% (w/v) sucrose, 6.0 ml of 27% sucrose, and 2.0 ml of 20% sucrose in Beckman SW 27.1 polyallomer centrifuge tubes. The six gradients were then centrifuged at 96,000 × *g* for 2.5 h. The turbid material which appeared at the 20%/27% and the 27%/32% sucrose interfaces (fractions I and I_1, respectively) was collected, each diluted 2-fold with homogenization buffer and centrifuged at 40,000 rpm in a Beckman Ti 50 rotor for 00 min.

Plasma Membrane Solubilization—The pellets of Fraction *I* (6 × 10^8 cell equivalents) were resuspended in 1.3 ml of 1.5% (w/v) *n*-octyl glucoside (Sigma) made in PBS and were incubated at room temperature for 60 min. The solution was centrifuged again in a Ti 50 rotor at 40,000 rpm for 60 min, and the supernatant containing the soluble plasma membrane protein was removed. The procedure was repeated on the pellets, and the resulting supernatants were combined with the previous supernatants. This detergent solution (approximately 2.5 ml) was dialyzed against 2 × 500 ml of PBS overnight and against a 1/5 dilution of PBS (in distilled water) for 1.5 h before being shell frozen and lyophilized. PC12 cells (1 × 10^8) yielded between 1.3 and 2.5 mg of soluble protein as determined by the protein assay of Bradford (22). Lyophilized material was redissolved in water immediately before immunization of animals.

Immunization of Mice—Female Balb/C mice (9-week-old) were injected subcutaneously with 700 μg of soluble protein from PC12 membranes (Fraction I) in incomplete Freund's adjuvant containing 2×10^9 inactivated *Bordatella pertussis*. Total injection volume was 200 μl. Booster injections were given at week 8 (4.2 mg of soluble protein in 120 μl of PBS) by intraperitoneal injection and at week 11 (3.75 mg of soluble protein in 200 μl of PBS) by intraperitoneal injection (100 μl) and intrasplenic injection (100 μl).

Fusion of Immune Splenocytes with Sp2/0 Myeloma Cells—Two days before fusion was performed, Sp2/0-Ag14 myeloma cells were subcultured to obtain cultures of low density (8 × 10^4 cells/ml). Three days after the final booster injection (11 weeks plus 3 days) spleens were removed aseptically from immune mice and splenocytes collected. Erythrocytes were lysed with two washes in 144 mM NH_4Cl, 17 mM Tris·Cl, pH 7.2 (23). Splenocytes (1.4 × 10^8) were fused with 1.4 × 10^8 Sp2/0 cells using the polyethylene glycol technique as described by Oi and Herzenberg (20). Cells were plated into 24-well plates (2-cm^2 wells, Co-star) at a density of 3 × 10^6 cells/well in a volume of 0.5 ml of HAT medium (see "Cell Culture" above). After 14 days, most wells had 2 to 5 colonies. Supernatants from each well were tested for activity in a screening assay (see below), and wells of interest were expanded in HT medium (HAT medium minus aminopterin) and subsequently cloned by limiting dilution into 96-well plates containing Balb/C thymocyte feeder layers. Clones were retested for activity, and ones of interest were expanded and frozen for storage.

Screening Assay—Supernatants from initial fusion wells were tested 14 days after fusion. Only those wells whose supernatants affected β-NGF binding to PC12 cells were kept. The assay was performed as follows. 100 μl of hybridoma supernatant or 100 μl of PBS-BSA were mixed with 100 μl of PC12 cells (4 × 10^6 cells/ml) and incubated on ice for 30 min in polystyrene culture tubes (12 × 75 mm, Falcon Plastics, Oxnard, CA). A volume (200 μl) of either ^{125}I-β-NGF (3.85 nM) or ^{125}I-β-NGF (3.85 nM) plus unlabeled β-NGF (385 nM) was added to bring the total volume to 400 μl. Specifically bound β-NGF was determined as described for binding assays (see below). Wells were expanded and cloned if the specifically bound β-NGF differed substantially between tubes which contained hybridoma supernatant and tubes which contained PBS-BSA (control binding). Final PC12 cell concentration in the assay was 1 × 10^6 cells/ml, final hybridoma supernatant dilution was 1/4, and final ^{125}I-β-NGF concentration was 1.92 nM.

β-NGF Preparation—β-NGF was prepared from submandibular glands of adult male Swiss-Webster mice (Simonsen, Gilroy, CA) by the method described by Varon *et al.* (24).

Iodination of β-NGF and 192-IgG—β-NGF was iodinated by the lactoperoxidase method described in detail by Sutter *et al.* (7). Specific activity of ^{125}I-β-NGF ranged from 1500–2500 cpm/fmol. ^{125}I-192-IgG was prepared using the chloramine-T method of Hunter (25) as modified by Aharonov *et al.* (26). A sample (300 μg) of 192-IgG was iodinated with a resulting speeific activity of approximately 350 cpm/fmol. Carrier-free $Na^{125}I$ was purchased from Amersham Corp. (IMS 30).

Binding Assays—Binding assays were conducted using either ^{125}I-β-NGF or ^{125}I-192-IgG as ligands. In general, PC12 cells were washed two times on tissue culture dishes with PBS-BSA before being mechanically dislodged by a sharp blow to the side of the dish. Cells were washed one additional time by centrifugation in PBS-BSA and were resuspended in PBS-BSA at 4 × 10^6 cells/ml. For all binding assays (except for time course experiments), 100 μl of cell suspension were preincubated with 100 μl of PBS-BSA (control) or 100 μl of monoclonal antibody solution (experimental) for 30 to 90 min on ice in polystyrene culture tubes. A volume (200 μl) of ^{125}I-labeled ligand (either β-NGF or 192-IgG) was then added at 2 times the final desired concentration, and incubation was continued on ice for an additional 90 min. At least 100-fold excess unlabeled ligand was included in parallel tubes to determine nonspecific binding. Specific binding is defined as the difference in binding between total and nonspecific binding. Cell-associated ligand was assayed by layering a 100-μl sample onto 175 μl of a 0.15 M sucerose solution (in PBS-BSA) in a 400-μl microfuge tube (Robbins Scientific Co., Mt. View, CA). These tubes were centrifuged for 1 min before being frozen in an ethanol/dry ice bath. Cells and bound ligand pelleted to the tip of the tube while free ligand stayed in the tube top. Tips containing the cell pellet (10^6 cells) were cut from the frozen tubes and counted by γ scintillation spectroscopy to determine the amount of cell-bound ligand. Tops were counted to determine free ligand concentration at the time of separation.

Slow β-NGF binding was assessed as described by Landreth and Shooter (11). 400 μl of cell-ligand mixture was cooled to 0.5 °C in the presence of 100-fold excess unlabeled ligand for 30 min. Cell-associated ligand (as measured in tube tip) after this 30-min incubation is defined as slow β-NGF binding.

192-IgG Production and Purification—Hybridoma clone 192-IgG grows well as an ascites tumor in Balb/C mice. Female Balb/C mice older than 5 months were injected intraperitoneally with 0.5 ml of

Pristane (2,6,10,14-tetramethylpentadecane; Aldrich). One to three weeks later, $4–6 \times 10^6$ 192-IgG hybridoma cells were injected intraperitoneally into each mouse. After 1–2 weeks, the ascites fluid containing 1–5 mg/ml of 192-IgG antibody was collected into heparinized tubes via a 16-gauge needle inserted into the abdominal cavity. The collected ascites fluid was centrifuged in a clinical centrifuge at 500 × *g* for 10 min to remove cellular elements. The supernatant was collected and made to 0.02% (w/v) in NaN_3. The pooled ascites fluid was dialyzed overnight against 50 mM Tris-Cl, 150 mM NaCl, 0.02% NaN_3, pH 8.4, at 4 °C. Because 192-IgG is an IgG_1 (see below) which does not bind to protein A, the dialyzed ascites fluid was loaded onto an anti-IgG column (constructed by covalently attaching 20 mg of goat anti-mouse IgG (Antibodies, Inc., Davis, CA) to 1 g of CNBr-activated Sepharose 4B (Sigma)) which had been equilibrated with the same buffer used for dialysis. Following sample loading, the column was washed with the same buffer until the absorbance at 280 nm (1 cm) dropped below 0.02 unit. Bound antibody was eluted with 0.1 M Na acetate buffer, pH 4.0, into tubes containing 0.5 M phosphate buffer, pH 7.4. Antibody fractions were combined, dialyzed against CMF-PBS, concentrated via ultrafiltration (XM50 filter, Amicon, Lexington, MA), centrifuged to remove particulates, aliquoted, and stored either at 4 or −20 °C. Antibody concentration was determined by absorbance at 280 nm using an extinction coefficient Σ^{1cm}_{280} of 1.38. Purified antibody was used for experiments presented in Figs. 2*B* and 3–6.

Antibody Class and Subclass Determination—Warm 1% (w/v) agar (Nobel agar, Difco) was poured onto GelBond plastic film (Bio-Products Dept., FMC Corp., Rockland, MD) and was allowed to solidify. Patterns of wells (15–20 μl volume) were punched into the agar. Dilutions of ascites fluid containing 192-IgG were added to central wells while various dilutions of rabbit anti-mouse immunoglobulins (anti-IgG, IgA, IgM from Miles-Yeda, Elkhart, IN; anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, from Litton Bionetics, Kensington, MD) were placed in surrounding wells. The plates were allowed to develop at room temperature in humidified chambers. Precipitin lines were evident at 24 h (class determination) or 48 h (subclass determinations).

Test for Direct Interaction of 192-IgG with β-NGF—Two methods were used. In the first a polyvinylchloride 96-well round bottom radioimmunoassay plate (Dynatech, Alexandria, VA) was coated with 25 μl of a 100 μg/ml solution of protein A (Sigma) for 1 h at room temperature after which wells were washed three times with PBS. Rabbit anti-mouse IgG (50 μl, undiluted, Miles-Yeda) was added for 1.5 h followed by three washes with PBS. 25 μl of monoclonal antibody (1/20 dilution of ascites fluid for 192-IgG and 151-IgG (50–250 μg/ml); undiluted culture supernatant for MC-β1 (50–60 μg/ml, obtained from hybridoma cells produced by Zimmermann *et al.* (27))) were added for an additional 1.5 h, followed by three washes with PBS-BSA. In control experiments using 10 μg/ml of ^{125}I-labeled monoclonal antibody, this protocol immobilized 22 fmol of monoclonal antibody per well (data not shown). A volume (25 μl) of ^{125}I-β-NGF (1.92 nM) or ^{125}I-β-NGF plus unlabeled β-NGF (192 nM) was added to various wells and incubation continued for 1.5 h at room temperature. Wells were then washed three times with PBS-BSA and were cut from the plate with a hot wire before being counted in a γ counter.

The second assay used to test for the possibility that 192-IgG directly binds β-NGF was performed using conditions which were similar to those in the cell-binding assays. 400 μl of ^{125}I-labeled MC-β1 or 192-IgG (300 nM and 1 μM, respectively) were incubated on ice with 40 μl of PBS-BSA or 40 μl of unlabeled β-NGF (3.7 μM, final concentration) for 30 min. Then, 40 μl of a 1:1 slurry of Sepharose 4B (Sigma) or 40 μl of a 1:1 slurry of Sepharose 4B-immobilized β-NGF (constructed using CNBr-activated Sepharose 4B; calculated β-NGF concentration on the Sepharose was approximately 60 pmol/40 μl of slurry) were added to the above mixtures and incubation was continued on ice for an additional 2 h. Sepharose was separated from the ^{125}I-labeled antibody mixture by a rapid centrifugation as described in the procedures for cell-binding assays. Antibody bound to the Sepharose or Sepharose-β-NGF was assayed in a γ counter.

Affinity Labeling Protocol—Cross-linking of ^{125}I-β-NGF and ^{125}I-192-IgG to PC12 cells was performed according to Massagué *et al.* (13), with the following modifications. PC12 cells (2×10^6 cells/ml) in 20 mM Hepes/Krebs-Ringer saline, pH 7.3, containing 1 mg/ml of both D glucose and bovine serum albumin were incubated for 60 min on ice with ^{125}I-labeled ligands in the absence or presence of unlabeled ligands at the indicated final concentrations. At the end of this period, cells were cooled to 0.5 °C, and cross-linking was started by diluting a freshly made stock of HSAB (5 mM in dimethyl sulfoxide) 1:100 into the cell-ligand mixture (final HSAB concentration was 50 μM) with gentle stirring. HSAB was obtained from the Pierce Chemical Co. This suspension was incubated for 3 min on ice in the dark and then photolyzed for 7 min in a quartz cuvette in the cold with a 200-watt Hg Oriel high pressure arc lamp equipped with a 330-nm cutoff filter. Photolysis was stopped by dilution into excess 10 mM Tris/saline, pH 7.0. The cells were pelleted at 1000 × *g* for 5 min, washed once more with the same medium, and the pellet was retained for electrophoresis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Affinity labeled washed PC12 cells were solubilized by boiling for 5 min in 58 mM Tris-Cl, pH 6.8, 1% sodium dodecyl sulfate, and 50 mM dithiothreitol. Samples (100 μg of protein) were subjected to electrophoresis on a 6% polyacrylamide slab gel according to Laemmli (28). After electrophoresis, the gels were stained in 0.1% Coomassie blue, 25% 1-propanol, 10% acetic acid and destained in 10% methanol, 10% acetic acid. Autoradiographs were obtained from the dried gels after a 10 to 20 days exposure to Kodak X-OMAT R film with enhancing screen (DuPont Lightning Plus). Protein standards used in a parallel run were: myosin (M_r = 200,000), β-galactosidase (M_r = 116,000), phosphorylase *b* (M_r = 94,000), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 45,000), and carbonic anhydrase (M_r = 31,000).

β-NGF-induced Neurite Regeneration Bioassay—The bioassay for NGF utilizing PC12 cells was described by Greene (29) and was modified as follows. PC12 cells were grown on 60-mm tissue culture plates (Falcon, Oxnard, CA) at an initial density of $4–5 \times 10^5$ cells/plate with 1.92 nM β-NGF added every second day for 8 days. Cells were removed sterilely from the dishes by gentle trituration with a pasteur pipette. This procedure also mechanically shears neurites from cell bodies. Cells were washed by centrifugation (3 min at 500 × *g*) once in fresh serum-containing growth medium and twice in serum-free growth medium in order to remove β-NGF. Cells were resuspended at 6×10^5 cells/ml in serum-free growth medium, and 0.5 ml was plated into each well of 24-well tissue culture plates which had been treated previously with 50 μg/ml of poly-L-lysine (Sigma) for 45 min at room temperature followed by 4 washes with sterile glass-distilled water. Cells were allowed to attach to the wells in a tissue culture incubator at 37 °C for 30 min, and then 400 μl of Dulbecco's modified Eagle's medium containing bovine serum albumin was added to bring the final serum albumin concentration to 1 mg/ml. Cells were placed in the tissue culture incubator for an additional 15 min before the addition of β-NGF (various concentrations) and/or other effectors. Plates were incubated in a tissue culture incubator for 24 h and then were scored by examination under a phase contrast microscope for the percentage of cells or cell aggregates which exhibited neurites at least 2 cell body diameters (greater than 25 μm) in length. Each condition was done in quadruplicate and at least 200 cells/well were counted.

RESULTS

Hybridoma Production—Immunization of mice with solubilized proteins from isolated plasma membranes of PC12 cells yielded immune splenocytes which, when fused with Sp2/0 myeloma cells, generated a hybridoma clone producing an antibody which interacted with β-NGF binding to PC12 cells. Mice were also immunized with whole PC12 cells or with isolated plasma membranes from PC12 cells. Fusions using these splenocytes yielded no hybridomas producing antibody which interacted with β-NGF binding, although greater than 80% of the initial fusion wells produced antibodies which bound to the surface of PC12 cells (data not shown).

192-IgG was selected for study because, in the initial screening of more than 600 supernatants, only the supernatant from this one well increased the specific binding of β-NGF to PC12 cells at subsaturating β-NGF concentrations (1.92 nM).

The concentration of antibody produced by this clone in serum-free medium (HB101) was low (approximately 3 μg/ml as determined by radioimmunoassay, data not shown) although cell growth was good. These cells produced ascites fluid in older Balb/C mice which had antibody concentration of 1–5 mg/ml. Induction of ascites fluid by these cells

younger mice (3 months old) was not as good, as a larger number of mice developed solid rather than ascites tumors. The effects of dilutions of culture supernatant and ascites fluid from clone 192-4-IgG on β-NGF binding to PC12 cells is shown in Fig. 1. The dilutions needed to achieve 50% of the maximal effect were approximately 1000-fold different, in close agreement with radioimmunoassay estimates of the antibody concentrations in these preparations.

192-IgG Characterization—A double radial diffusion assay was used to determine the antibody class and subclass of 192-IgG. A range of dilutions was used for both the ascites fluid and the class-specific antisera. The precipitin line which formed was sharpest at a 1/10 dilution of ascites fluid and undiluted rabbit anti-mouse IgG antiserum. The precipitin line in the subclass determination took longer to form (48 instead of 24) and was sharpest at a 1/50 dilution of ascites fluid and undiluted rabbit anti-mouse IgG_1 (data not shown). Antisera against IgA, IgM, and IgG subclasses 2a and 2b did not form precipitin lines with 192-IgG ascites fluid.

192-IgG Does Not Directly Bind β-NGF—A possible explanation for the increase in β-NGF binding to PC12 cells observed in the initial screening assays and in Fig. 1 was that the antibody was able to directly bind β-NGF both on the cell surface and in solution. This possibility was tested by attaching 192-IgG to a polyvinylchloride well and then incubating the well with ^{125}I-β-NGF, 192-IgG did not specifically bind β-NGF (Fig. 2*A*) although a control monoclonal antibody (MC-β1) had this ability. Likewise, a monoclonal antibody (151-IgG) which interacts with epidermal growth factor binding did not bind β-NGF in this experiment.

In another experiment designed to approximate the conditions of our screening and binding assays, β-NGF which had been immobilized on Sepharose 4B was not able to bind ^{125}I-192-IgG (Fig. 2*B*). A control antibody which was made against β-NGF was bound by this β-NGF-Sepharose preparation. The specificity of this binding was determined in parallel reactions which contained free β-NGF as well as the β-NGF-Sepharose. This free β-NGF was able to compete for binding of ^{125}I-MC-β1 but had no effect on the small amount of binding of ^{125}I-192-IgG. The nonspecific binding of ^{125}I-192-IgG was to Sepharose rather than to the β-NGF in the β-NGF-Sepharose preparations as is shown in control experiments also presented in Fig. 2*B*.

These experiments exclude the possibility that the effect of 192-IgG on β-NGF binding to PC12 cells is due to a direct interaction with the ligand.

192-IgG Increases the Apparent Affinity of β-NGF Binding—It was noted during screening assays that the effect of 192-IgG on β-NGF binding was greater at lower concentrations of β-NGF. This suggested that the increase in binding could be due to an alteration in the dissociation constant (K_D)

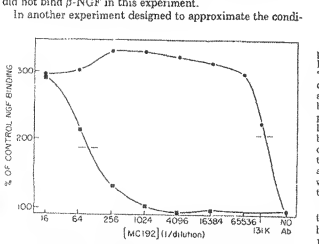

FIG. 1. **192-IgG increases β-NGF binding to PC12 cells at 0.5 °C.** PC12 cells were mechanically dislodged from tissue culture dishes by several sharp blows to the sides of the dishes. The cells were washed two times by centrifugation in PBS-BSA before being resuspended at approximately 4×10^6 cells/ml in PBS-BSA. Cells and all reagents were cooled to 0.5 °C before use. 100 μl of cell suspension were added to 100 μl of antibody (*Ab*) solution (4 times final concentration) and mixed gently. 30 min later, 200 μl of ^{125}I-β-NGF (2 times final concentration) were added, and incubation was continued for another 60 min. Cells with bound β-NGF were separated by a rapid centrifugation step as described under "Materials and Methods." Final cell concentration: 1×10^6/ml; final ^{125}I-β-NGF concentration: 0.96 nM; final antibody dilution: reciprocal shown on *x* axis. Data are presented as the percentage of control NGF binding (no antibody present). 100% = 4564 cpm (ascites curve), = 7605 cpm (culture supernatant curve). Nonspecific binding has been subtracted from each point and was less than 10% of control binding in all cases. Each *point* represents the mean of triplicate samples. Standard deviations were less than 10% of the value in all cases. ■, culture supernatant; ●, ascites fluid.

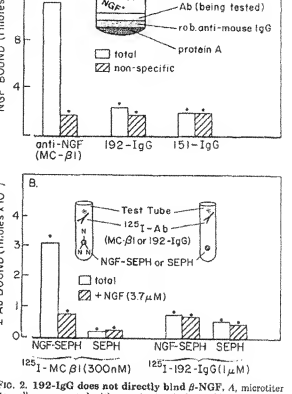

FIG. 2. **192-IgG does not directly bind β-NGF.** *A*, microtiter plate wells were coated with protein A solution, rabbit anti-mouse IgG antibody, and monoclonal antibody (*Ab*) as described under "Materials and Methods." ^{125}I-β-NGF (1.92 nM) was added and incubated at room temperature for 1.5 h. Nonspecific binding was assayed in the presence of 192 nM unlabeled β-NGF. Values shown by *bars* are the mean of duplicate wells while the greater of the two points measured are indicated by ●. *PVC*, polyvinylchloride. *B*, ^{125}I-labeled antibody was incubated with either PBS-BSA or with unlabeled β-NGF for 30 min on ice. Either β-NGF-Sepharose (*SEPH*) (4B or Sepharose 4B was added and incubation continued for an additional 2 h. The Sepharose was separated from the reaction mixture as indicated under "Materials and Methods," and bound antibody was determined by measuring ^{125}I in a γ counter. Values shown are the mean of quadruplicate points ± S.D.

of β-NGF for one or both of its cellular receptors. β-NGF binding assays were performed in the presence or absence of 192-IgG (67 nM) in order to measure the K_D of β-NGF binding and to measure the number of binding sites for β-NGF on PC12 cells. ^{125}I-β-NGF concentrations ranged from 0.045 to 23 nM. Assays were performed on ice to minimize the contribution of sequestration, internalization, and degradation in an effort to meet the criteria needed for steady state binding. The results are presented as binding isotherms in Fig. 3*A* and as a Scatchard analysis in Fig. 3*C*. The measured K_D for β-NGF in this experiment was 3.2 nM without 192-IgG and 1.3 nM in the presence of 192-IgG, an increase in affinity of 2.5-fold in the presence of 192-IgG. In similar experiments, the measured increase in affinity has been as great as 4-fold. The 192-IgG, therefore, increases the affinity of the fast NGF-R. In the presence and absence of 192-IgG, the number of β-NGF binding sites was approximately 30 fmol/10^6 cells or 180,000 receptors/cell. This experiment does not show whether 192-IgG affects slow binding. The cause of the deviation from linearity in the Scatchard plot for β-NGF in the presence of 192-IgG (Fig. 3*C*, *open circles*) is not known.

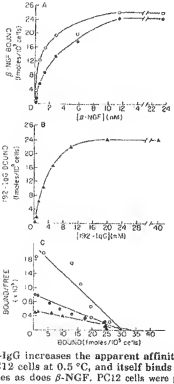

FIG. 3. **192-IgG increases the apparent affinity of β-NGF binding to PC12 cells at 0.5 °C, and itself binds to the same number of sites as does β-NGF.** PC12 cells were prepared and binding assays were performed as described for Fig. 1. Cells were incubated with PBS-BSA or with 192-IgG for 30 min before ^{125}I-labeled ligand (β-NGF or 192-IgG), or ^{125}I-labeled ligand plus a 500-fold excess of unlabeled ligand was added. After addition of ligand, cells were incubated for an additional 120 min on ice. Triplicate samples were assayed for bound ligand as described under "Materials and Methods." Nonspecific binding has been subtracted from all points. Standard deviations are less than 10% of the values shown. *A*, β-NGF binding isotherm. β-NGF specific activity = 2418 cpm/fmol ●, ^{125}I-β-NGF alone; ○, ^{125}I-β-NGF plus 192-IgG (67 nM, final concentration); *B*, 192-IgG binding isotherm. ▲, ^{125}I-192-IgG alone. 192-IgG specific activity = 353 cpm/fmol. *C*, data of *A* and *B* presented as Scatchard plots. Regression lines were drawn by the method of least squares. ●, ^{125}I-β-NGF, $r = -0.991$; ○, ^{125}I-β-NGF plus unlabeled 192-IgG (67 nM final concentration), $r = -0.949$; ▲, ^{125}I-192-IgG, $r = -0.968$.

192-IgG and β-NGF Bind to the Same Number of Sites at 0.5 °C on PC12 Cells—If 192-IgG is an anti-NGF-R antibody, it should bind to the same number of sites on PC12 cells as does β-NGF. A binding assay utilizing ^{125}I-192-IgG was performed using concentrations of antibody from 0.08 to 40 nM. Again, this experiment was performed at 0.5 °C to reduce the complications inherent at higher temperatures. A time course of binding of 192-IgG to PC12 cells shows that greater than 90% of equilibrium binding had been reached by 120 min when 192-IgG concentration was 3 nM. At a higher concentration of 192-IgG, 100% of the equilibrium binding had been reached by this time (data not shown). Fig. 3*B* shows the binding isotherm for 192-IgG and Fig. 3*C* contains the Scatchard analysis of the data. In this assay, PC12 cells bound approximately 32 fmol of 192-IgG/10^6 cells, an estimate which is in close agreement with the amount of β-NGF bound. The calculated K_D for 192-IgG binding was approximately 6 nM. When β-NGF was present (7.7 nM), the K_D for 192-IgG binding was shifted to 4.8 nM, a slightly higher affinity. The number of binding sites for 192-IgG did not change in the presence of β-NGF (data not shown). These results also exclude the possibility that 192-IgG is binding directly to β-NGF.

192-IgG Retards the Appearance of Slow β-NGF Binding at 37 °C—Binding to the fast NGF-R which is the major class observed at 0.5 °C was affected by the inclusion of 192-IgG (Fig. 3*A*). It was of interest to examine the effect of 192-IgG on slow NGF binding. This slow binding is best observed at an incubation temperature of 37 °C and at low β-NGF concentrations (11). Both 192-IgG and ^{125}I-β-NGF were preincubated with PC12 cells at 0.5 °C in order to establish equilibrium binding conditions before inducing the appearance of the slow binding component by shifting of the incubation temperature to 37 °C. It was found that 192-IgG retarded the appearance of slow β-NGF binding while enhancing the total amount of β-NGF bound (Fig. 4). In the presence of the antibody the ratio of fast to slow binding was much greater than in its absence even after 90 min of incubation.

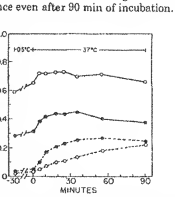

FIG. 4. **192-IgG slows the formation of slow β-NGF binding at 37 °C.** PC12 cells were prepared and resuspended at approximately 4×10^6 cells/ml as described in Fig. 1. 1.8 ml of cells were added to 1.8 ml of 192-IgG (133 nM), and the mix was incubated on ice for 30 min. 3.6 ml of ^{125}I-β-NGF (38 pM) or 3.6 ml of ^{125}I-β-NGF plus unlabeled β-NGF (38 nM) were added, and incubation was continued on ice for an additional 60 min. Points shown for $t = -30$ and $t = 0$ were sampled during this incubation. The tubes were shifted to 37 °C at $t = 0$, and triplicate samples were taken for each time point shown to measure total binding and slow binding as described under "Materials and Methods." Data are presented as femtomoles of β-NGF bound to approximately 10^6 cells. Standard deviations are less than 5% of the mean for all points. Final ^{125}I-β-NGF concentration = 19 pM; final 192-IgG concentration = 33.25 nM. β-NGF specific activity = 2418 cpm/fmol. ●, β-NGF alone; ○, β-NGF plus 192-IgG; ——, total binding; - - -, slow binding.

The incubation of PC12 cells with 0.15 nM ^{125}I-NGF followed by photolysis in the presence of the heterobifunctional photoactivatable cross-linker, HSAB, has previously been shown to result in the labeling of only one membrane component which migrates on polyacrylamide gels containing sodium dodecyl sulfate at a position corresponding to an M_r of 158,000 (30). However, at 0.8 nM ^{125}I-β-NGF, an additional component with M_r of 100,000 was found to be labeled when the same cross-linking protocol was used (31). This is similar to the results obtained with sympathetic neuronal membranes, where two labeled components with M_r of 143,000 and 112,000, respectively, were also observed when ^{125}I-β-NGF was covalently cross-linked to them (13). On the basis of the differential susceptibility of the two labeled bands to dissociation in the presence of excess unlabeled β-NGF, the M_r = 158,000 species was identified as the slow receptor (chase stable) and the smaller M_r = 100,000 species as the fast receptor (chase labile) (31). As demonstrated in Figs. 3 and 4, incubation of PC12 cells with 192-IgG resulted in increased binding of ^{125}I-β-NGF to the fast receptor. Therefore, the presence of 192-IgG during incubation with ^{125}I-β-NGF should lead to a selective increase in the labeling of the M_r = 100,000 (fast) receptor species. Fig. 5 shows that the labeling of this species was indeed increased, when 192-IgG at 33.3 nM was present during the incubation with 0.1 nM ^{125}I-β-NGF (*lane 2*), as compared to a control with no antibody present (*lane 1*). A concomitant decrease in the labeling of the M_r = 158,000 slow receptor species in the presence of 192-IgG was also noted. At low temperature, the antibody has no direct effect on binding of NGF to the slow receptor. The decreased binding of NGF to this receptor can be explained by the observed increase in the affinity of the fast receptor, which at the β-NGF concentration used, causes a substantial reduction in the amount of free NGF. Preincubation of PC12 cells with 192-IgG prior to the addition of ^{125}I-β-NGF did not increase the labeling of the M_r = 100,000 component (data not shown).

The same cross-linking protocol was used to investigate the nature of the protein on the surface of PC12 cells to which 192-IgG binds, in particular to investigate whether it interacts with a component of the NGF receptor(s) or with yet another protein. When PC12 cells were incubated for 60 min on ice with 6.67 nM ^{125}I-192-IgG, a variety of protein bands was found to be labeled (Fig. 5*B*, *lane 4*). To distinguish between bands arising from covalent cross-linking of ^{125}I-192-IgG to component(s) of PC12 cells and those merely representing intra- or intermolecular cross-linking adducts of the antibody which had not been washed out, ^{125}I-192-IgG was cross-linked with HSAB in the absence of PC12 cells. After eliminating long-lived reactive photolysis products of HSAB by incubation in the presence of 10 mM Tris-Cl, pH 7.0, for 60 min on ice, the cross-linked antibodies were added to PC12 cells, and incubation was continued for another 60 min on ice. Comparison of the labeling pattern obtained under these conditions (Fig. 5*B*, *lane 3*) to that obtained when PC12 cells were photolyzed in the presence of both ^{125}I-192-IgG and HSAB (Fig. 5*B*, *lane 4*) shows that under the latter incubation conditions, only one major band with M_r of 160,000 originates from cross-linking of ^{125}I-192-IgG to PC12 cells. All the others (with lower M_r) were also found in the prephotolysis control (*lane 3*) and most likely represent cross-linking adducts of ^{125}I-192-IgG which had not been washed out.

The observation that the labeling of this M_r = 160,000 band was not affected by the presence of excess β-NGF (data not shown) has no significance since binding of 192-IgG to PC12

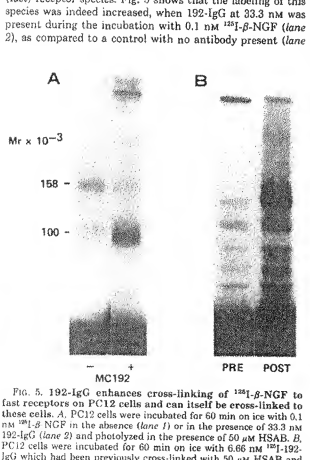

FIG. 5. 192-IgG enhances cross-linking of ^{125}I-β-NGF to fast receptors on PC12 cells and can itself be cross-linked to these cells. *A*, PC12 cells were incubated for 60 min on ice with 0.1 nM ^{125}I-β-NGF in the absence (*lane 1*) or in the presence of 33.3 nM 192-IgG (*lane 2*) and photolyzed in the presence of 50 μM HSAB. *B*, PC12 cells were incubated for 60 min on ice with 6.66 nM ^{125}I-192-IgG which had been previously cross-linked with 50 μM HSAB and incubated with 10 mM Tris-Cl, pH 7.0, for 60 min on ice (*lane 3*) or with fresh ^{125}I-192-IgG (*lane 4*). For *lane 4*, cross-linking with HSAB was performed as described under "Materials and Methods." The washed cell pellets were solubilized and samples (100 μg of cell protein) were electrophoresed on the same 6% polyacrylamide gel as indicated under "Materials and Methods." Autoradiographs of portions of the fixed dried gels are shown. The M_r of the cross-linked components are indicated on the *left*.

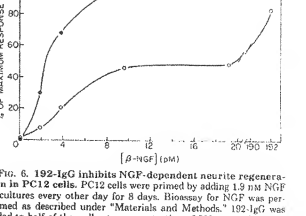

FIG. 6. 192-IgG inhibits NGF-dependent neurite regeneration in PC12 cells. PC12 cells were primed by adding 1.9 nM NGF to cultures every other day for 8 days. Bioassay for NGF was performed as described under "Materials and Methods." 192-IgG was added to half of the wells at a concentration of 300 nM, and β-NGF was added to all wells at the concentrations indicated except for control wells. Results are presented such that 100% = maximal response observed (45% of the initial cells responding) and 0% = basal neurite formation in the absence of added β-NGF (3.5% of the initial cells responding). Standard deviations are less than 10% of the mean for all points. Each *point* represents the mean of quadruplicate wells with at least 200 cells counted per well. ●, β-NGF alone; ○, β-NGF plus 192-IgG (300 nM).

cells is not competed by β-NGF.

192-IgG Inhibits NGF-dependent Neurite Regeneration in PC12 Cells—Primed PC12 cells on poly-L-lysine regenerate neurites only in the presence of β-NGF (29). This provides a quantitative assay for measuring the dose-response relationship of β-NGF with PC12 cells. The antibody 192-IgG decreased the percentage of cells which regenerated neurites over a range of β-NGF concentrations from 2 to 19 pM (Fig. 6). The cells not bearing neurites remained "phase-bright," and indeed at higher β-NGF concentrations (192 pM), the percentage of cells regenerating neurites in the presence of 192-IgG approached the control values. These observations are consistent with the hypothesis that 192-IgG is exerting its effect by altering β-NGF binding rather than by decreasing the cell viability.

DISCUSSION

Sympathetic and some embryonic sensory neurons as well as PC12 cells exhibit two classes of NGF receptors (11, 12). A comparison of the dose-response curves of NGF-induced neurite outgrowth from sensory neurons with receptor occupancy suggests that this biological response correlates with the occupancy of the higher affinity receptor (9). The same holds for PC12 cells (32). Whether the slow receptors preexist on PC12 cells as suggested by Schechter and Bothwell (12) or are formed as a result of ligand first binding to fast receptors, followed by conversion to the slow form (11), is still not resolved.

The monoclonal antibody 192-IgG increases the binding of β-NGF to the fast receptors of PC12 cells at both low temperature and 37 °C. The fact that the same number of β-NGF and 192-IgG molecules bind to PC12 cells at low temperature supports the idea that 192-IgG is an antibody directed, at least, against the fast receptor. However, it is still possible that the antibody recognizes another cell-surface protein present in equal numbers to the fast receptor and is interacting with it. Attempts to identify directly the antigen recognized by 192-IgG by a protein transfer technique (33) have thus far proved unsuccessful. In an alternative method, the cross-linking of ^{125}I-192-IgG to PC12 cells revealed only one major specifically labeled band with M_r of 160,000. However, since β-NGF does not inhibit 192-IgG binding to PC12 cells it is still not proven that this band reflects cross-linking of ^{125}I-192-IgG to one of the β-NGF receptors. The band does have a molecular weight consistent with the cross-linking of one heavy and one light chain of 192-IgG (M_r of 75,000 for the combined chains) to the fast receptor of M_r = 85,000 (the M_r = 100,000 complex less one β chain). The M_r of bands which appear after photolysis in the absence of PC12 cells are consistent with those expected from various cross-linked adducts of heavy and light antibody chains and BSA.

The enhancement of binding to the fast receptor was due to a 2.5 to 4-fold increase in the affinity of β-NGF and not to a change in receptor number (Fig. 3, *A* and *C*). The 192-IgG did not affect the amount of β-NGF finally bound to slow receptors at 37 °C although it did affect the rate of appearance of binding to this component (Fig. 4). This latter effect could be due either to a decreased rate of association of β-NGF with pre-existing slow receptors (12) or to inhibition of the rate of conversion of fast to slow receptors (11) by, for example, steric hindrance, or both.

The finding that at low temperature 192-IgG increased the formation of the M_r = 100,000 complex previously identified with ^{125}I-β-NGF cross-linked to the fast receptor (31) further corroborates the finding that 192-IgG enhances binding of β-NGF to the fast receptor. At this temperature, the observed decrease in the formation of the M_r = 158,000 complex in the presence of antibody could be explained by the decreased availability, at any given β-NGF concentration, of β-NGF to bind to the slow receptor because of the increased affinity of the fast receptor. Alternatively the antibody may also inhibit conversion of fast to slow receptors. At 37 °C, at low concentrations of β-NGF, the antibody inhibits neurite outgrowth from PC12 cells while at high β-NGF concentrations no decrease of biological activity is observed. This effect could again be explained by either interference with the conversion of fast to slow receptors or by decreased binding to the slow receptor at low but not high β-NGF concentrations. Both explanations implicate the slow receptor as the key mediator of the biological action of NGF. However, the explanation is not as simple as this because the temperature-jump experiment showed that the amount of slow binding finally achieved in the presence of antibody was at least as great as that in the absence of antibody. It appears that it is not simply the extent of slow receptor occupancy which determines the biological response but rather the kinetics with which this occupancy occurs. The 192-IgG antibody may prove to be a useful tool in defining these kinetic requirements as well as defining secondary causal steps after β-NGF binding in the pathway leading to neurite regeneration.

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Potent Human p140-TrkA Agonists Derived from an Anti-Receptor Monoclonal Antibody

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Monoclonal antibody (mAb) 5C3 directed against human p140 TrkA is a structural and functional mimic of nerve growth factor (NGF) and an artificial receptor agonist. mAb 5C3 binds in the NGF-docking site and, like NGF, it promotes TrkA internalization, TrkA and phosphatidylinositol-3 kinase tyrosine phosphorylation, and increased transformation of TrkA-expressing fibroblasts. More important, mAb 5C3 protects human TrkA-expressing cells from apoptotic death in serum-free media. Interestingly, agonistic activity is observed with monomeric F_{ab} 5C3 fragments. mAb 5C3 ($K_d \sim 2$ nM) was used to study features of ligand binding by TrkA and the distribution of TrkA protein in normal human brain.

Key words: NGF; receptor; TrkA; agonist; antibody; ligand

The TrkA receptor is a 140 kDa transmembrane glycoprotein with tyrosine kinase activity that functions as the nerve growth factor (NGF) receptor (Kaplan et al., 1991; Klein et al., 1991). NGF also binds with low affinity to a p75 receptor the signaling function of which is unclear (Chao, 1992). Homodimers of TrkA or heterodimers of TrkA and p75 bind NGF with higher affinity (Hempstead et al., 1991; Jing et al., 1992; Mahadeo et al., 1994), suggesting that specific receptor conformations may play specific functions.

TrkA protein or mRNA are expressed in neural crest-derived sensory and sympathetic neurons, possibly in cholinergic neurons (Cavicchioli et al., 1991), within the basal forebrain and striatum (Holtzman et al., 1992; Verge et al., 1992), and in some non-neuronal tissues (Chevalier et al., 1994). Functional studies of neuronal cultures *in vitro* have suggested that TrkA protein is expressed throughout the cell surface (Campenot et al., 1994). However, whether this also is true within the architecture of the brain remains to be established.

NGF promotes the differentiation of certain neuronal cells, is mitogenic for TrkA-transfected fibroblasts, and allows survival in serum-deprived conditions for both cell types. Activation of the tyrosine kinase activity of TrkA via NGF binding leads to receptor trans- and auto-tyrosine phosphorylation (PY), and PY of second messengers including phosphatidylinositol-3 kinase (PI-3 kinase) (Soltoff et al., 1992). PI-3 kinase is involved in protein trafficking and endocytosis of ligand–receptor complexes (for review, see Kaplan and Stephens, 1994). Because microinjection of NGF into cells does not induce NGF biological signals (Heumann et al., 1984), cell-surface receptor ligation and internalization of TrkA or NGF–TrkA complexes must mediate these effects.

TrkA, like most kinase growth factor receptors, signals through receptor oligomerization (Heldin, 1995). Thus, monovalent TrkA-binding agents are antagonistic or have no biological effects (Clary et al., 1994; LeSauteur et al., 1995), whereas bivalent receptor-binding agents such as NGF (a homodimer; Bradshaw et al., 1993) or antibodies can be agonistic. The principle of using polyclonal antibodies to activate neural receptors has been demonstrated previously (Clary et al., 1994; Twyman et al., 1995). In contrast, only a limited number of anti-receptor monoclonal antibodies mimic ligand functions (Galloway et al., 1992; Taub and Greene, 1992), and none exists against neurotrophin receptors.

In this study, we report the development and characterization of an agonistic anti-human TrkA mAb 5C3 that recognizes the NGF-docking site. mAb 5C3 was used to characterize the pattern of TrkA protein expression in normal human brain and the NGF-binding features of the receptor. mAb 5C3 behaves like NGF in bioassays, and monomeric 5C3 F_{ab}s retained binding and functional agonistic activity. mAb 5C3 will be useful to identify the NGF-docking site on TrkA and possibly as a pharmacological lead in the development of small mimetics.

MATERIALS AND METHODS

Antibodies

Female Balb/c mice were immunized with Balb/c-3T3 cells transfected with human TrkA, and splenocytes were fused to SP2/0 myelomas by the general method of Geller (1977). Hybridomas were screened by differential binding between untransfected and TrkA-transfected cells using a FACScan (Becton Dickinson, San Jose, CA) (see below). mAb 5C3 [IgG1(κ)] was identified and subcloned three times. Rat anti-mouse IgG (emIgG; Sigma, St. Louis, MO), anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY), and anti-PI-3 kinase polyclonal serum (Upstate Biotechnology) were purchased commercially, mouse anti-rat p75 mAb MC192 ascites were a gift from P. Barker, and anti-p65 mAb 87.92.6 (Co et al., 1985) was grown in our laboratory.

Monomeric mAb 5C3 F_{ab}s

mAb 5C3 was purified (1 mg/ml) with Protein G–Sepharose (Sigma) and digested with papain (10 μg/ml; Gibco, Toronto, Ontario, Canada) as described previously (Coligan et al., 1991). F_{ab}s were repurified on

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KappaLock-Sepharose (Upstate Biotechnology) and Protein G–Sepharose and dialyzed against PBS. All products were characterized by SDS-PAGE under nonreducing or reducing conditions (100 mM 2-mercaptoethanol) to >98% purity (data not shown). Control F_{ab}s from anti-rat p75 mAb MC192 were prepared similarly.

Cell lines

Mouse SP2/0 myelomas, mouse R1.1 and EL4 thymomas, mouse NIH-3T3 fibroblasts, mouse 2B4 T cell hybridomas, NGF-responsive rat PC12 pheochromocytoma cells, human Jurkat T lymphomas, and human HeLa fibroblasts were used. NIH-3T3 cells transfected either with human p140*trkA* cDNA (E25 cells), with p75 cDNA (Z91 cells), or with p75 and p140*trkA* cDNAs (R7 cells) were provided by Dr. M. Barbacid (Jing et al., 1992). The *trk*-negative rat B104 neuronal cell line (expressing endogenous rat p75) and B104-transfected with human *trkA* cDNA (4-3.6 cells, expressing human TrkA and rat p75) were provided by Dr. E. Boggenmann (Boggenmann et al., 1995). All cells were cultured in RPMI media supplemented with 5–10% fetal bovine serum (FBS) and antibiotics (Gibco). Transfectants were added the appropriate drug selection.

FACScan

Cells (5×10^5) in 0.1 ml of binding buffer [HBSS, 0.1% bovine serum albumin (BSA), and 0.1% NaN_3] were incubated with the indicated concentration of mAbs or F_{ab}s for 30 min at 4°C, washed in binding buffer to remove excess primary antibody, and immunostained with fluorescein-ated [fluorescein isothiocyanate (FITC)] goat anti-mouse IgG (FITC-GamIgG), or anti-mouse Fab (FITC-GamF_{ab}s; Sigma) secondary antibody for 30 min at 4°C ([Bandoola et al., 1993). Cells were acquired and analyzed on a FACScan using the LYSIS II program. As negative controls (background fluorescence), mouse IgG (Sigma), mAb 192, or 192 F_{ab}s were used as primary, followed by appropriate secondary.

Biochemical analysis

Cell lysates. For cell lysates, 33×10^6 cells/ml were detergent-solubilized (lysis buffer 2% Nonidet P-40, 150 mM NaCl, 50 mM Tris-glycine, 10 mM NaF, 50 μM Na_2VO_4, 30 mM Na-pyrophosphate, 10 mM benzamidine, and 20 mM iodoacetamide, pH 7.8) supplemented with protease inhibitors (2 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) for 30 min at 4°C, followed by a 15 min centrifugation at 14,000 × *g*. Cleared supernatants were analyzed by SDS-PAGE directly (whole-cell lysates) or after immunoprecipitation.

Gel analysis. Cell lysates were prepared in Laemmli electrophoresis sample buffer and analyzed by SDS-PAGE under reducing (100 mM 2-mercaptoethanol) or nonreducing conditions. Prestained protein markers (Gibco) were used as reference. Protein concentrations were quantitated by the biuret assay (Bio-Rad, Melville, NY) and by parallel Coomassie blue staining of SDS-PAGE gels. For Western blotting, samples were electrotransferred to polyvinylidene difluoride (Xymotech Biosystems, Mt. Royal, Montréal, Québec, Canada), blocked overnight in TBST (0.05 M Tris base, 0.2 M NaCl, 0.5% Tween-20, pH 7.6) containing 1% BSA (Sigma), and immunoblotted with the indicated primary mAbs. Secondary antibodies were either horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (HRP-GαR) or goat anti-mouse IgG (HRP-GαM; Sigma). For detection the enhanced chemiluminescence (ECL) reagents (Amersham, Oakville, Ontario, Canada) were used according to the manufacturer's instructions. Densitometric analysis was performed with a Masterscan interpretive densitometer (Scanalytics, Billerica, MA) and a Scanmaster (Howtek, Hudson, NH).

Binding, competition, and internalization assays

mAb 5C3 was ^{125}I-labeled by the Iodogen (Pierce, Rockford, IL) method (Harlow and Lane, 1988) to a specific activity of 1.8 mCi/mg. [^{125}I]5C3 was repurified from free ^{125}I with Sephadex G25 columns (15×1 cm^3) to >96% trichloroacetic acid-precipitable incorporation. Binding studies were performed with serial dilutions of [^{125}I]5C3 on 0.5×10^6 E25 or 4-3.6 cells (and their respective controls, NIH-3T3 and B104 cells; data not shown) for 1 hr at 4°C. Cell-associated [^{125}I]5C3 and free [^{125}I]5C3 were counted after washing unbound ligand. Parallel [^{125}I]NGF (70 mCi/mg; DuPont NEN, Mississauga, Ontario, Canada)-binding assays were performed as control (data not shown). Competition of [^{125}I]5C3 binding was done in binding assays in the presence of unlabeled mAb 5C3 (100-fold molar excess) or unlabeled NGF (500-fold molar excess, saturating concentration).

Competition of [^{125}I]NGF binding was performed by first incubating cells with excess unlabeled mAb 5C3, NGF, mAb 87.92.6, or vehicle-binding buffer for 30 min at 4°C. [^{125}I]NGF then was added to a final saturating concentration of ~1 nM, the mixtures were incubated for an additional 45 min at 4°C, cells were washed, and cell-associated [^{125}I]NGF was determined.

For receptor internalization studies, cells were incubated with TrkA-binding agents (0.01 μg of mAb 5C3, 2 nM NGF) or controls (mIgG, HBSS) for 20 min either at 37°C (internalization-permissive temperature) or at 4°C (internalization-nonpermissive temperature). After washing, cells were processed for surface TrkA immunofluorescence with mAb 5C3 primary and FITC-GamIgG secondary as described above and analyzed by FACScan.

Proliferation/survival assays

Cells (5000 cells/well) in serum-free media (SFM; Gibco) supplemented with 0.1% BSA were added to 96-well plates (Falcon, Lincoln Park, NJ) containing serial dilutions of NGF, mAb 5C3, control mAbs, mAb 5C3 F_{ab} fragments, control mAb 192 F_{ab} fragments (data not shown), or serum (final 5% FBS, normal growth conditions). Where indicated, F_{ab}s were externally cross-linked with goat anti-mouse F_{ab} (GαMF_{ab}s; Sigma). Wells containing all culture conditions but no cells were used as blanks. The proliferative/survival profile of the cells was quantitated using the tetrazolium salt reagent 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) 48–72 hr after plating as initially described by T. Mosmann (Hansen et al., 1989). Optical density readings of MTT were done in an EIA Plate Reader model 2550 (Bio-Rad) at 600 nm with the blanks subtracted. Assays were repeated at least five times in quadruplicates.

Foci-formation assays

E25 cells (15×10^3) were plated in a 25% serum containing 0.35% soft *agar mixture in the presence of* mIgG *control* (0.5 μg/ml), mAb 5C3 (0.5 μg/ml), or NGF (2 nM). Conditions were replenished every 3 d, and foci were counted after 2 weeks.

Immunocytochemistry of human brain tissues

Human brain tissue was obtained from six males (age 71.7 ± 4.6 years) without signs of neurological or psychiatric disorders. Tissue blocks were prepared (mean time postmortem 16.2 ± 3.5 hr) and stored at −80°C. Cryostat sections (20 μm thick) were fixed (4% *p*-formaldehyde, 0.1 M phosphate, pH 7.4, for 1 hr at 4°C) and rinsed in PBS for 1 hr at 4°C. Immunocytochemistry was performed using avidin–biotin complex (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) as described previously (Hsu et al., 1981). Primary mAb 5C3 was used either as a 1:1000–1:4000 dilution of ascites or a 1:4 dilution of SFM culture supernatant. Nickel ammonium sulfate (0.5%) was used to amplify the signal in the diaminobenzidine revelation step. Some sections also were stained with cresyl violet to facilitate the cytoarchitectural analysis. Negative controls were performed without primary antibody or with normal mouse IgG as primary and, in all cases, yielded no detectable immunolabeling.

RESULTS

Characterization of mAb 5C3

To assess mAb 5C3 specificity for human TrkA, cells expressing or lacking TrkA were screened for differential binding by FACScan analysis measuring cell-associated fluorescence. Binding of mAb 5C3 to nonpermeabilized TrkA-expressing cells demonstrated that it recognizes the extracellular domain of human TrkA (Table 1, Fig. 1*A*). Human TrkA transfectant lines 4-3.6, E25, and R7 bound mAb 5C3. In contrast, rat PC12 (expressing rat TrkA and rat p75), rat B104 (parental cells of 4-3.6, expressing rat p75), Z91 (NIH-3T3 transfected with p75), wild-type NIH-3T3, or NIH-3T3 cells transiently transfected with human *trkB*, rat *trkA*, or rat *trkB* cDNA did not bind mAb 5C3. Thus, mAb 5C3 is specific for human TrkA, and coexpression of rat or human p75 does not interfere with binding.

The concentration of mAb 5C3 required to saturate TrkA receptors in E25 cells was determined by testing increasing amounts of antibody in FACScan assays (Fig. 1*A*). Receptor

Table 1. Surface phenotyping with mAb 5C3

Cells	5C3 Binding
E25 (hTrkA)	++++
RJ (hTrkA/p75)	+++
Z91 (p75)	–
4-3.6 (hTrkA/p75)	+++
B104 (p75)	–
PC12 (rTrkA/p75)	–
Transient NIH-3T3 Transfections	
htrkA cDNA	++
htrkB cDNA	–
rtrkB cDNA	–
rtrkA cDNA	–

The indicated cell lines expressing human TrkA (hTrkA), rat TrkA (rTrkA), and/or p75 were analyzed by surface immunofluorocytometry with mAb 5C3 versus control mIgG. Transient transfections (48 hr) were done by electroporation of cDNAs (provided by M. Chao). Relative intensities of staining are indicated as ++++ (high staining) or – (no staining, equivalent to background fluorescence; see Fig. 1). Saturating doses of mAb were used, and differences represent receptor number. Other cells tested include wild-type NIH-3T3, Jurkat, R1.1, EL4, 2B4, and HeLa cells, which are all negative (data not shown).

saturation is evident at 2 μg/ml mAb 5C3, at which concentration the fluorescence intensity is maximal. Similar analysis with mAb 5C3 F_{ab}s demonstrated that specificity (data not shown) and saturability were similar to that obtained with intact mAb. Lower F_{ab} protein concentrations (0.7 μg/ml) were required for receptor saturation (Fig. 1*B*). Because the molecular weight of 5C3 F_{ab} is threefold lower than 5C3 IgG (~50 vs ~150 kDa, respectively), equimolar concentrations of IgG and F_{ab} ligands were required to saturate hTrkA.

Western blot analysis with mAb 5C3 revealed heterogeneous material of M_r 140 kDa (p140) for samples from E25 and 4-3.6 cells but not for control cells (Fig. 2*A*). In these cells, a band of ~110 kDa (p110) was also observed, previously thought to be intracellular TrkA precursors (Martín-Zanca et al., 1989). The p140 band also was immunoblotted in samples dissected from normal human cortex or nucleus basalis of Meynert (Fig. 2*B*). The p110 band was not seen, perhaps because of different post-translational processing in neuronal tissues with respect to transfected cell lines. mAb 5C3 was effective in Western blot analysis only when samples were prepared under nonreducing conditions, indicating that a disulfide bond-stabilized conformational epitope is recognized.

Immunostaining in normal human brain

mAb 5C3 was used to map TrkA protein expression by immunocytochemistry of normal adult human brains. The striatum, basal forebrain, and brainstem exhibited the strongest immunostaining, whereas only weak staining could be detected in the cerebral cortex and hippocampal formation (Fig. 3).

All sectors of the basal nucleus contained large TrkA-positive neurons (Fig. 3*A*,*C*), most of them in groups embedded in a dense network of overlapping stained processes (Fig. 3*A*). The cells had heterogeneous shapes, ranging from complex multipolar to fusiform.

In the basal ganglia, TrkA was detected in distinct cellular compartments. The caudate nucleus, nucleus accumbens, and putamen contained several immunoreactive cell bodies without apparent distinction in density, perikaryal staining, or shape. Figure 3*D* shows typical labeled multipolar neurons that displayed strong granular immunoreactivity around the nucleus and in proximal processes. Moreover, numerous puncta and varicose fiber fragments were observed in these areas. The globus pallidus and claustrum were mostly negative except for varicose fibers. Similarly, the interstitial elements and fiber bundles did not contain reactive fibers, whereas the internal capsule displayed some labeled punctuas and fibers, particularly near the putamen and caudate nucleus.

The hippocampal formation showed weak immunostaining located principally in scattered fibers and puncta in the stratum granulosum of the dentate gyrus, as well as in the strata oriens and pyramidale of Ammon's horn. In addition, some weakly stained perikarya could be observed in the stratum pyramidale of the CA2 and CA3 subfields of Ammon's horn and in the hilus of the dentate gyrus (CA4 subfield; Fig. 3*E*). The

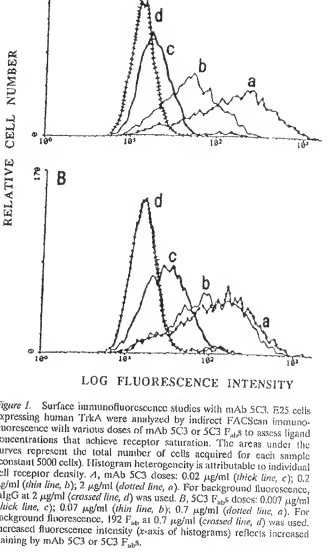

Figure 1. Surface immunofluorescence studies with mAb 5C3. E25 cells expressing human TrkA were analyzed by indirect FACScan immunofluorescence with various doses of mAb 5C3 or 5C3 F_{ab} to assess ligand concentrations that achieve receptor saturation. The areas under the curves represent the total number of cells acquired for each sample (constant 5000 cells). Histogram heterogeneity is attributable to individual cell receptor density. *A*, mAb 5C3 doses: 0.02 μg/ml (*thick line, c*); 0.2 μg/ml (*thin line, b*); 2 μg/ml (*dotted line, a*). For background fluorescence, mIgG at 2 μg/ml (*crossed line, d*) was used. *B*, 5C3 F_{ab}s doses: 0.007 μg/ml (*thick line, c*); 0.07 μg/ml (*thin line, b*); 0.7 μg/ml (*dotted line, a*). For background fluorescence, 192 F_{ab} at 0.7 μg/ml (*crossed line, d*) was used. Increased fluorescence intensity (*x*-axis of histograms) reflects increased staining by mAb 5C3 or 5C3 F_{ab}s.

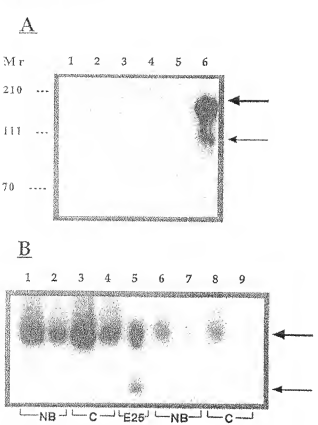

Figure 2. Direct detection of p140 TrkA by Western blotting. Whole-cell detergent lysates (2×10^6 cell equivalents/lane) were resolved by SDS-PAGE under nonreducing conditions and analyzed by Western blotting with mAb 5C3. *A*: *Lane 1*, Jurkat; *lane 2*, PC12; *lane 3*, NIH-3T3; *lane 4*, RJ.1; *lane 5*, Z91; *lane 6*, E25. *Thick arrow*, p140 TrkA; *thin arrow*, p110. *B*, Dissected human brain tissues: nucleus basalis (*NB*; *lanes 1, 2, 6, 7*) and cortex (*C*; *lanes 3, 4, 8, 9*) were compared to E25 cells (*lane 5*; 2×10^6 cell equivalents). *Lanes 1, 3*, 300 μg/lane; *lanes 2, 4*, 150 μg/lane; *lanes 6, 8*, 75 μg/lane; *lanes 7, 9*, 33 μg/lane. *Thick arrow*, p140 TrkA; *thin arrow*, p110. Note that p110 is not seen in the human brain tissues.

perikarya of these neurons were relatively large, of ovoid to pyramidal shape, and bearing one prominent apical and radial dendritic process. The immunoreactivity appeared, as in other stained cell types of the brain, as small granular patches of precipitate located principally near the nuclear envelope and in some cases within the cytoplasm (Fig. 3*E*).

Within the cerebral cortex, particularly in the frontal area, TrkA immunoreactivity appeared more discrete. At high magnification, immunoreactive puncta and fiber fragments without a particular pattern of distribution are observed in all layers, but laminae III–VI appeared more stained than superficial ones (Fig. 3*H*). Weakly staining, medium-sized perikarya were occasionally observed in layer IV (Fig. 3*H*).

In the brainstem, TrkA staining also is detected. The pontine nuclei contained numerous immunoreactive, medium-sized globular perikarya and fibers between the pontocerebellar fibers (Fig. 3*F*). The reticular formation also displayed strong immunoreactivity for TrkA principally located in fiber networks (Fig. 3*G*). Some large neurons of bipolar or multipolar shape also are stained. No TrkA immunostaining was observed in the cerebellum (not shown).

Binding studies

Scatchard plot analysis of [^{125}I]5C3-binding assays demonstrated that in the E25 cell surface there are ~250,000 5C3-binding sites/cell with a K_d of 1.6 ± 1.0 nM (Fig. 4), and in the 4-3.6 cell surface there are ~200,000 5C3-binding sites/cell with a K_d of 3.0 ± 2.0 nM (data not shown). No [^{125}I]5C3 binding was observed for parental NIH-3T3 or B104 cells (data not shown). Competition with saturating concentrations of NGF reduced the number of 5C3-binding sites in E25 cells by ~25%. However, NGF caused no detectable changes in the affinity of mAb 5C3 for TrkA receptors. Similar data were obtained measuring mAb 5C3-binding sites by FACScan analysis, in which a decrease was observed after NGF treatment (see Table 3).

In the converse experiment, mAb 5C3 inhibited ~60% of [^{125}I]NGF binding to E25 cells. In these experiments, background binding was assessed by blocking with 5 μM NGF (100% inhibition), and maximal binding was assessed with binding buffer vehicle only (0% inhibition) or by using irrelevant binding mAb 87.92.6 (Table 2).

Functional agonism of mAb 5C3

Several functional assays of NGF bioactivity were used to test the agonistic potential of mAb 5C3.

Receptor internalization

The 4-3.6 cells were treated with TrkA ligands at internalization-permissive temperatures (37°C) or at nonpermissive temperatures (4°C; Table 3). NGF treatment reduced the percent staining of mAb 5C3 to surface TrkA at both temperatures. Loss of surface 5C3-binding sites suggests direct blocking by NGF (see also Fig. 4). In contrast, mAb 5C3 treatment reduced the number of surface 5C3-binding sites only at 37°C. This is likely attributable to receptor internalization, which does not occur efficiently at 4°C. Treatment with mIgG or binding buffer control did not reduce the number of surface 5C3-binding sites at either temperature. Similar data were obtained with E25 cells (data not shown).

Receptor PY

Anti-phosphotyrosine Western blots of E25 or 4-3.6 whole-cell detergent extracts revealed that TrkA PY increased significantly over basal levels after short treatment with mAb 5C3 or with NGF (Fig. 5). Densitometric analysis of several blots from E25 and 4-3.6 cells is presented in Table 4. Other proteins, including ~95 and ~60 kDa proteins and the p85 subunit of PI-3 kinase (~2.5-fold increase; data not shown), also showed increased PY. We have estimated that <10% of all p85 material was tyrosine-phosphorylated after ligation of TrkA.

Increased cellular transformation

NGF treatment causes the transformation and an increase in anchorage-independent growth of TrkA-expressing E25 cells (Cordon-Cardo et al., 1991). mAb 5C3 caused an approximately twofold increase in the number and size of foci compared with mIgG-treated cells (Table 5). No change in the number or size of foci was observed in wild-type NIH-3T3 cells after mAb 5C3 treatment (data not shown).

Protection from cell death

Agonistic ligands of TrkA protect receptor-expressing cells from death in SFM. Both NGF and mAb 5C3 increased the number of

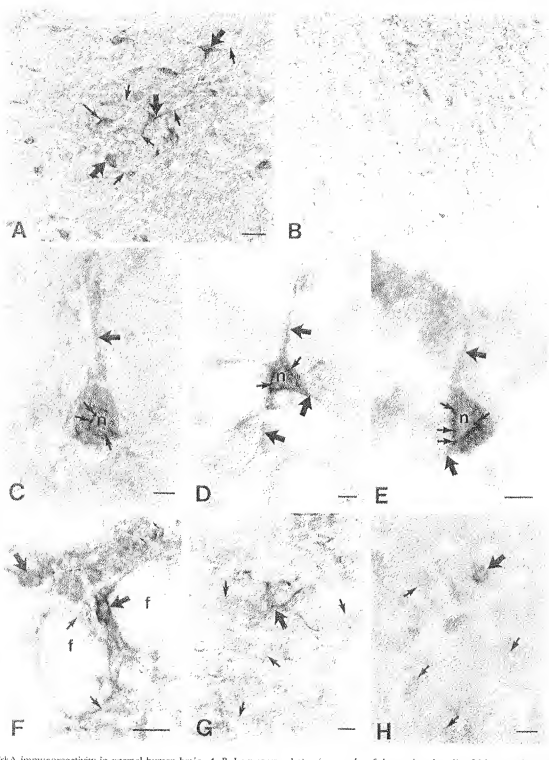

Figure 3. TrkA immunoreactivity in normal human brain. *A, B*, Low-power photomicrographs of the nucleus basalis of Meynert show large neurons (*arrows*) immunoreactive with mAb 5C3 (*A*) but lacking immunoreactivity with normal mouse IgG (*B*) in a consecutive section. Note in *A* that the labeled neuronal processes can often be followed (*small arrows*). *C–E*, High-power photomicrographs of TrkA-containing neurons in the nucleus basalis (*C*), the putamen (*D*), and the CA4 subfield of the hippocampus (*E*). The perinuclear area displayed particularly strong concentration of diaminobenzidine precipitate (*small arrows*), often in granules. Labeled proximal processes also could be observed (*arrows*); *n*, nucleus. *F*, In the pontine nuclei, many weakly to strongly staining neurons (*arrows*) are observed within the fiber network (*small arrows*) and around the nonlabeled fiber bundles (*f*). *G*, In the reticular formation of the brainstem, numerous fibers (*small arrows*) constitute a network in which some scattered neurons (*arrow*) are observed. *H*, Photomicrograph of TrkA immunoreactivity in the frontal cerebral cortex showing weak labeling. A few neurons are weakly positive (*arrows*), with the staining residing mainly in puncta, possibly corresponding to fibers (*small arrows*). Scale bars: *A, B*, 50 μm; *C–E, H*, 10 μm; *F, G*, 20 μm.

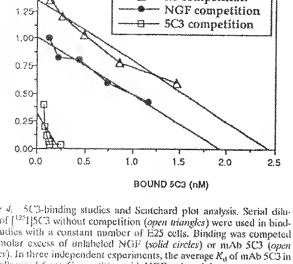

Figure 4. 5C3-binding studies and Scatchard plot analysis. Serial dilutions of [^{125}I]5C3 without competition (*open triangles*) were used in binding studies with a constant number of E25 cells. Binding was competed with molar excess of unlabeled NGF (*solid circles*) or mAb 5C3 (*open squares*). In three independent experiments, the average K_d of mAb 5C3 in E25 cells was 1.6 nM. Competition with NGF reduced the average number of 5C3-binding sites, but the affinity of mAb 5C3 was not affected.

Table 2. mAb 5C3 blocks NGF binding to TrkA

Treatment	% NGF binding
mAb 5C3	39.3 ± 7.4
mAb 87.92.6	100
NGF (5 μM)	0

E25 cells expressing TrkA (but not p75 receptors) were incubated with [^{125}I]NGF in the presence of the indicated agents. [^{125}I]NGF binding after treatment with mAb 87.92.6 was identical to treatment with vehicle binding buffer. Assays were done three times in duplicate. Data are expressed as percent binding ± SD, where mAb 87.92.6 is maximum and 5 μM NGF is background binding as per the formula: [(test − background) ÷ 100%]/(maximum − background).

Table 3. mAb 5C3-induced TrkA-receptor internalization

Treatment	Temperature (°C)	% 5C3 staining
NGF (2 nM)	4°C	83 ± 2.0
	37°C	75 ± 3.6
5C3 (0.01 μg/ml)	4°C	96 ± 0.0
	37°C	77 ± 5.5

TrkA surface immunostaining was performed on 4-3-6 cells with mAb 5C3 after the indicated treatments and measured by FACScan analysis. Data are presented as percent staining ± SEM, with reference to control vehicle treatment (100%) as per the following formula: [(treated sample staining − mfgG background staining) ÷ 100%]/(maximum staining − mfgG background staining).

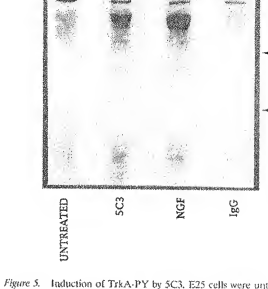

Figure 5. Induction of TrkA-PY by 5C3. E25 cells were untreated (*lane 1*) or treated with mAb 5C3 (*lane 2*), NGF (*lane 3*), or mIgG (*lane 4*) for 15 min at 37°C. Whole-cell lysates were resolved in an 8% SDS-PAGE under reducing conditions and immunoblotted with anti-phosphotyrosine mAb 4G10. A parallel gel under nonreducing conditions immunoblotted with mAb 5C3 (not shown) controlled for M_r and equal loading of TrkA on all samples.

surviving/proliferating E25 fibroblastoid cells (Fig. 6). Equivalent protection also was afforded by TrkA ligands to neuronal 4-3-6 cells (data not shown). In most experiments, mAb 5C3 protection is dose-dependent, although high-dose antibody inhibition sometimes is seen (e.g., 1 μg/ml mAb 5C3).

To ascertain whether cell death is apoptotic, DNA was prepared from serum-free cultured cells that showed a typical apoptotic fragmentation ladder. The DNA ladder was not seen in preparations from cells cultured in the presence of mAb 5C3 or NGF (data not shown).

Controls demonstrated the functional specificity of mAb 5C3. First, neither NGF nor mAb 5C3 protected wild-type NIH-3T3 cells (data not shown). Second, PC12 cells were not protected by mAb 5C3 but were protected by NGF (data not shown). Third, irrelevant mfgG, GoatF$_{ab}$, or mAb 192 did not protect E25 cells (Fig. 6) or NIH-3T3 cells (data not shown).

Functional agonism of monomeric 5C3 F$_{ab}$s

Monovalent agents that bind TrkA behave as competitive antagonists (Clary et al., 1994; LeSauteur et al., 1995) likely because they cannot induce receptor dimerization. Therefore, it would be expected that monomeric 5C3 F$_{ab}$s would be monovalent and not be able to mediate agonistic function.

mAb 5C3 F$_{ab}$s afforded protection from apoptotic death to E25 cells (Fig. 6) and 4-3-6 cells (data not shown) in SFM. Moreover, anti-phosphotyrosine Western blots revealed that cells treated with 5C3 F$_{ab}$s had increased TrkA-PY similar to increases obtained with whole mAb 5C3 (data not shown).

Monomeric 5C3 F$_{ab}$ protection was dose-dependent. However, equivalent or better protective effects were achieved when F$_{ab}$s were cross-linked externally with GoatF$_{ab}$ antibodies. Specificity controls included those described in the previous section for whole mAb 5C3, plus 192 F$_{ab}$s that had no protective activity in E25 cells (data not shown).

DISCUSSION

The availability of antibodies against p140 TrkA and p75 has allowed the study of these NGF receptors (Martin-Zanca et al., 1989; Eager, 1991). The mAb 5C3 reported in this study is specific for human TrkA and functions in FACScan immunofluorescence analysis, immunoprecipitation, Western blot analysis, and immu-

Table 4. Increased TrkA-PY by mAb 5C3

Treatment	E25 cells	4-3.6 cells
mAb 5C3	2.7 ± 0.6	3.4 ± 1.5
NGF	6.5 ± 1.3	3.8 ± 0.8

E25 or 4-3.6 cells were untreated or treated with saturating concentrations of mAb 5C3 or NGF for 15 min at 37°C. Whole-cell lysates or anti-PY immunoprecipitates were resolved by SDS-PAGE under reducing conditions, Western-transferred, immunoblotted with anti-phosphotyrosine mAb 4G10, and developed using ECL techniques. Optical density readings were taken from x-ray films with film background subtracted (see Materials and Methods). Data are presented as fold increase in PY of TrkA with respect to untreated cells ± SD; $n = 3$.

Table 5. MAb 5C3-induced anchorage-independent growth

Treatment	Average number of foci[a]	Typical cells/foci[a]	Fold increase in foci[b]
mIgG	416 ± 45	~24	1 ± 0.11
mAb 5C3	806 ± 178	>48	1.9 ± 0.22
NGF	676 ± 51	~24	1.6 ± 0.08

E25 cells were cultured in soft agar in the presence of the indicated agents for 2 weeks.

[a]Average number ± SD and typical size of foci are shown.

[b]Fold increase in foci was calculated with respect to mIgG-treated cells (no increase); $n = 2$.

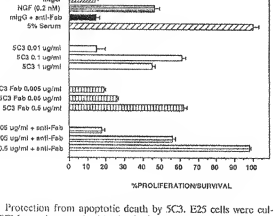

Figure 6. Protection from apoptotic death by 5C3. E25 cells were cultured in SFM supplemented with the indicated conditions for 2–3 d, followed by the MTT assay. Similar data were obtained with neuronal 4-3.6 cells (data not shown). The percent proliferation/survival ± SD was determined by standardizing serum-containing wells to 100% using the following formula: [(optical density of test) × 100%]/(optical density of serum).

nocytochemistry. Moreover, mAb 5C3 is a structural and functional mimic of NGF.

Aberrant expression of *trkA* mRNA and NGF responsiveness have been correlated with neurodegenerative disorders (for review, see Ebendal et al., 1991) and neoplastic malignancy (Marchetti et al., 1993; Matsushima and Bogenmann, 1993). Hence, TrkA-binding agents will be useful clinical tools in diagnosis, prognosis, and perhaps treatment of these diseases. Indeed, mAb 5C3 binding is a positive prognostic marker for certain human neoplasias (K. Kramer, unpublished observations).

mAb 5C3 was used to map the distribution of TrkA protein in the normal human brain postmortem. The data are consistent with the distribution of *trkA* mRNA and p140 TrkA protein previously described in numerous neurons of the basal forebrain and striatum (Holtzman et al., 1992; Steininger et al., 1993; Allen et al., 1994; Martinoff et al., 1994). Moreover, the present study has revealed TrkA immunostaining in other cell types of the human brain including the hippocampal formation, cerebral cortex, and brainstem.

The presence of equivalent levels of TrkA protein (per weight of tissue) in the cortex and the nucleus basalis of Meynert was further supported biochemically by Western blot analysis. Quantitative differences between *in situ* mRNA hybridization and immunostaining may reflect increased sensitivity of the mAb 5C3, long TrkA protein half-life, post-transcriptional control of expression, or instability of the mRNA.

Correlation between TrkA and choline acetyltransferase immunostaining (Mesulam and Geula, 1991; De Lacalle et al., 1994) suggest that most TrkA-labeled perikarya express the cholinergic phenotype. This was confirmed by studies of colocalization (Steininger et al., 1993; Martinoff et al., 1994). However, our results indicate that some TrkA-positive cells are not cholinergic, because the hippocampal formation does not contain intrinsic cholinergic cells in the human brain (De Lacalle et al., 1994).

mAb 5C3 recognizes a disulfide-stabilized domain of TrkA, and an extracellular epitope with these characteristics appears to be the NGF-docking site (Perez et al., 1995; Urfer et al., 1995). Cross-blocking studies indicated that mAb 5C3 and NGF can reciprocally block each other's binding to TrkA, suggesting further that the docking site of 5C3 may be similar to NGF. In addition, sequence comparison of both ligands revealed interesting homology between complementary determining regions (CDR) of mAb 5C3 and the variable-turn regions of NGF (S. Maliartchouk and H. Saragovi, unpublished observations). Because most CDR are β-turns (Sibanda et al., 1989) and, coincidentally, the NGF structures that bind TrkA also may be β-turns (LeSauteur et al., 1995), we hypothesized that both mAb 5C3 and NGF bind to the same site on human TrkA, and cross-blocking is likely to be caused by direct competition rather than steric hindrance.

Interestingly, mAb 5C3 was more efficient at blocking NGF binding than vice versa. Only ~25% of the mAb 5C3-binding sites on E25 fibroblasts were blocked by saturating doses of NGF. These data suggest that not all TrkA receptors in this transfected cell line bind NGF. It is unlikely that affinity considerations can account for these observations, because both ligands have roughly comparable K_d for TrkA (mAb 5C3, $K_d \sim 1.6$ nM; NGF, $K_d = 0.7$ nM; Jing et al., 1992) and the affinity of mAb 5C3 was unchanged in the presence of NGF.

Three nonexclusive possibilities can account for these observations: (1) TrkA receptors exist at equilibrium, at which ~25% are in an NGF-binding conformation (e.g., dimers) and the rest are in a non-NGF-binding conformation; (2) specific post-translational modifications of TrkA receptors allow for NGF binding; and/or (3) expression of other membrane proteins [e.g., p75 or an unknown protein(s)] induces or favors the NGF-binding conformation of TrkA. These hypotheses can be addressed by biochemical analysis after differential affinity purification of TrkA with mAb 5C3 versus NGF and by further binding studies in neuronal and fibroblastoid cells expressing different receptors.

The absence of mAb 5C3 binding to rat TrkA is intriguing. Binding by mAb 5C3 to rat TrkA was expected because of the homology between mAb 5C3 CDRs and the variable loops of NGF, particularly because NGF from one species does bind to TrkAs from other species. mAb 5C3 is a binding and structural

mimic of NGF, with enhanced human receptor specificity. Remodeling and mutating of the CDRs of mAb 5C3 will yield a pan-TrkA-binding mAb. Furthermore, analysis of the epitope of mAb 5C3 on TrkA likely will reveal differences in the docking sites of human and rat TrkAs. This information will be useful in screening receptor-binding analogs.

To test functional mimicry by mAb 5C3, NGF bioassays were performed using *trkA*-transfected fibroblast and neuronal cells. Functional mimicry by mAb 5C3 included TrkA internalization, TrkA-PY, PI-3 kinase PY, increased anchorage-independent growth, and proliferation/survival of cells in SFM. By these criteria, mAb 5C3 is agonistic.

Increased TrkA-receptor turnover or internalization is induced by NGF binding. mAb 5C3 increased the internalization of TrkA, as measured by loss of cell-surface receptors. These results are consistent with data that showed that E25 cells internalize I^{125}]NGF within seconds after shifting from 4 to 37°C (Jing et al., 1992) and that this process does not require p75 receptors. Thus, artificial ligands of TrkA can induce receptor internalization and could be useful in delivering toxic agents to the cytoplasma of TrkA-expressing tumors.

NGF bination of TrkA causes receptor activation and autophosphorylation. mAb 5C3 induced TrkA-PY to a similar degree. Agonism in the absence of NGF suggests that TrkA dimerization and/or internalization is the required signaling event, rather than the formation of NGF–TrkA complexes. However, we cannot rule out that mAb 5C3-TrkA may be the functional signal-transducing complex.

Ligand-induced PY of the intracellular domain of TrkA allows for the recruitment of substrates and the activation of cytosolic proteins and nuclear oncoproteins. mAb 5C3 induces the PY of proteins of M_r 60, 85, and 95 kDa. The 85 kDa protein was identified as PI-3 kinase, the activation of which correlates with the actions of growth factors and oncogenes.

NGF stimulates neuronal survival and differentiation (for review, see Barbacid, 1994) and the proliferation of non-neuronal cells (Marchetti et al., 1993). NGF-activated TrkA induces transformation and morphological changes in fibroblast cells (Cordon-Cardo et al., 1991). mAb 5C3 caused similar increases in anchorage-independent growth and foci formation in soft agar. Thus, mAb 5C3 can positively modulate the growth of TrkA-expressing cells. Interestingly, the size of the mAb 5C3-induced foci were larger on average than NGF-induced foci. We currently are investigating possibilities that may account for this observation.

TrkA-expressing neuronal 4-3.6 cells or fibroblastoid E25 cells undergo apoptotic death in SFM but can be rescued by NGF or mAb 5C3. Synergy between the two ligands occurred when combined at suboptimal doses (data not shown), as would be expected if mAb 5C3 bound and activated unoccupied TrkA receptors. Furthermore, morphological changes and increased attachment to plastic were observed in both NGF- and 5C3-treated cells.

Monomeric 5C3 F_{ab}s protected E25 and 4-3.6 cells from apoptotic death. When F_{ab}s were cross-linked externally using anti-F_{ab} antibodies, a heightened response occurred. Because growth factor–receptor activation requires bivalent binding (Clary et al., 1994; Heldin, 1995), the monomeric 5C3 F_{ab}s must have retained the ability to induce TrkA oligomerization. This could be explained in the following three ways: (1) F_{ab}s are relatively large molecules capable of aggregation; (2) 5C3 F_{ab} binding could cause conformational changes in TrkA that induce receptor–receptor interactions; and (3) monomeric 5C3 F_{ab}s bind to two receptor molecules in a bivalent manner. The last possibility could occur by two CDRs binding to two different TrkAs. Homology of mAb 5C3 CDRs to NGF turn regions and experiments using small recombinant antibody analogs (S. Maliartchouk and H. Saragovi, unpublished observations) support the third explanation.

mAb 5C3 is the first reported agonistic anti-neurotrophin receptor mAb and will be useful in studies of TrkA biology and for drug development. Anticoplastic effects with mAb 5C3 may be achieved through terminal differentiation, antibody-dependent cell cytotoxicity, or by the delivery of toxins or radionuclides. Furthermore, the structure of this mAb may be useful in designing peptidic and nonpeptidic TrkA-binding agents (Saragovi et al., 1991). Small, nonpeptidic agonists of TrkA should be useful pharmacological agents for the treatment of neurodegenerative diseases.

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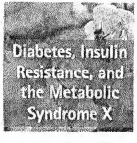

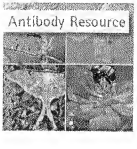

Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3)

Anti-IGF-R
Anti-Insulin-Like Growth Factor Receptor

Host: Mouse
Isotype: IgG_1

Immunogen: partially purified IGF-I receptor from human placenta
Form: Liquid
Formulation: In 0.05 M sodium phosphate buffer, 0.2% gelatin.
Preservative: ≤0.1% sodium azide
Positive Control: HepG2 cells
Negative Control: HS27 cells

Comments: Recognizes the ~130 kDa α and the ~90 kDa β subunits of IGF-I receptor.

Ref.: Roth, R. 1988. *Science* **239,** 1269. Rohlik, Q.T., et al. 1987. *Biochem. Biophy Res.* Rosen, O.M., 1987. *Science* **257,** 1452. Rechler, M.M. and Nissley, S.P., 1986. *Hormone* Ullrich, A., et al. 1986. *EMBO J.* **5,** 2503. Zapf, S. and Froesch, E.R., 1986. *Hormone Res* Humbel, R.E., 1984. I. *Chemistry; in LI Hormonal proteins and peptides.* Vol 12, Chap. 4 (New York). Kull, F.C., et al. 1983. *J. Biol. Chem.* **258,** 6561.

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EMD Chemicals Inc. USD list price is displayed (pricing with local distributors may vary). Nc is based on item availability worldwide.
Sales Office Contact Details

Size	In Stock	Qty
100 µg	Y	

Add to Cart

Clone	Species Reactivity	Applicatio
αIR3	human	IF, IP, NOT IB, Nt See Key

EMD Chemicals Inc. USD list price is displayed (pricing with local distributors may vary). Nc is based on item availability worldwide.
Sales Office Contact Details

Product Name	Cat. No.	Size	In Stock

Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3)	GR11T	10 µg	Y

Add to Cart

Related Products:

GR11: Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3)
GR11L: Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3)
GR11T: Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3)

Related Literature:

Diabetes and Obesity Brochure

Protein Kinase and Related Tools Brochure

Material Safety Data Sheets:

GR11: Anti-IGF-I Receptor (Ab-1) Mouse mAb (αIR3) - English

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Finnish	Hungarian	Polish	

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All Categories » Calbiochem » Antibodies » Primary Antibodies » Receptors » Growth Factor

Selected Citations:

1. Rita Nahta, et al. (2005) Insulin-like growth factor-I receptor/human epidermal gro receptor 2 heterodimerization contributes to trastuzumab resistance of breast can *Research* **65**, 11118-11128.
2. Daniel E. Syroid, et al. (1999) A role for insulin-like growth factor-I in the regulatio survival. *Journal of Neuroscience* **19**, 2059-2068.
3. Donald M. Henricks, et al. (1998) Identification of insulin-like growth factor I in bov plasma and its receptor on spermatozoa: influence on sperm motility. *Biology of F* 330-337.

Conforms to 91/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number

Customer Number

1. Identification of the substance/preparation and of the company/undertaking

Product name : IGF-1 Receptor (Ab-1) Monoclonal Antibody

Chemical formula : N/A

Synonym : Anti-IGF-R
Anti-Insulin-Like Growth Factor Receptor

Catalog # : GR11

Supplier : Manufactured by EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
(858)450-9558/(800)854-3417
FAX: (858)453-3552

Emergency telephone number : Call Chemtrec®
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name	CAS No.	EC Number	Symbol	R-Phrases
Anti-IGF-R Anti-Insulin-Like Growth Factor Receptor	N/A	Not available.	-	-

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : No specific hazard.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are nitrogen oxides (NO, NO2 ...).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions	:	Splash goggles. Full suit. Boots. Gloves. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.
Small Spill and Leak	:	Absorb with an inert material and put the spilled material in an appropriate waste disposal.
Large Spill and Leak	:	Absorb with an inert material and put the spilled material in an appropriate waste disposal.

7. Handling and storage

Handling	:	Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe gas/fumes/ vapor/spray.
Storage	:	Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store below 4°C (39.2°F).
Packaging materials		
Recommended use	:	Use original container.

8. Exposure controls/personal protection

Engineering measures	:	Provide exhaust ventilation or other engineering controls to keep the airborne concentrations of vapors below their respective threshold limit value. Ensure that eyewash stations and safety showers are proximal to the work-station location.
Hygiene measures	:	Wash hands after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
IGF-1 Receptor (Ab-1) Monoclonal Antibody	Not available.

Personal protective equipment

Skin and body	:	Lab coat.
Eyes	:	Safety glasses.
Protective Clothing (Pictograms)	:	

9. Physical and chemical properties

Physical state	:	Liquid.
Color	:	Not available.
Molecular Weight	:	Not available.
Solubility	:	Not available.
Flash point	:	Not available.
Explosive properties	:	Risks of explosion of the product in presence of mechanical impact: Not available. Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability	:	The product is stable.
Conditions to avoid	:	Not available.
Hazardous Decomposition Products	:	These products are nitrogen oxides (NO, NO2. .).

11. Toxicological information

RTECS #	:	N/A
Local effects		
Skin irritation	:	Not available.
Acute toxicity	:	LD50: Not available. LC50: Not available.
Chronic toxicity	:	Repeated or prolonged exposure is not known to aggravate medical condition.
Other Toxic Effects on Humans	:	Not available. No specific information is available in our database regarding the other toxic effects of this material for humans. Not available. To the best of our knowledge, the toxicological properties of this product have not been thoroughly investigated.

Carcinogenic effects	:	Not available.
Mutagenic effects	:	Not available.
Reproduction toxicity	:	Not available.
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available.
Toxicity of the Products of Biodegradation	:	The product itself and its products of degradation are not toxic.

13. Disposal considerations

Methods of disposal; Waste of residues; Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations.

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class	:	Not controlled under ADR (Europe).

Sea

IMDG Class	:	Not controlled under IMDG.

Air

IATA-DGR Class	:	Not controlled under IATA.
Special Provisions for Transport		Not applicable.

15. Regulatory information

EU Regulations

Risk Phrases	:	This product is not classified according to the EU regulations.
U.S. Federal Regulations	:	TSCA: No products were found. SARA 302/304/311/312 extremely hazardous substances: No products were found. SARA 302/304 emergency planning and notification: No products were found. SARA 302/304/311/312 hazardous chemicals: No products were found. SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found. SARA 313 toxic chemical notification and release reporting: No products were found. Clean Water Act (CWA) 307: No products were found. Clean Water Act (CWA) 311: No products were found. Clean air act (CAA) 112 accidental release prevention: No products were found. Clean air act (CAA) 112 regulated flammable substances: No products were found. Clean air act (CAA) 112 regulated toxic substances: No products were found.
HCS Classification	:	Not controlled under the HCS (United States).
State Regulations	:	
WHMIS (Canada)	:	Not controlled under WHMIS (Canada) No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

	0
Flammability	1
Reactivity	0
Personal Protection	A

National Fire Protection Association (U.S.A.)

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